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EFFECT OF NEONATAL INJECTION OF CHICKEN ERYTHROCYTES ON NATURAL HETERO-HEMAGGLUTININ TITER IN MICE[1]

D. R. ASHFORD,[2] F. M. FAIRFIELD,[3] AND G. O. BAIN

Abstract

Intravenous injection of mice within 6 hours of their birth with chicken erythrocytes resulted in depression of the titer of naturally occurring heteroagglutinins for chicken red cells. By the time the mice were 16 weeks of age, the titer had risen significantly but had not attained control levels. Subsequent challenge by multiple intraperitoneal injections of chicken red cells revealed that impairment of serological responsiveness to this antigen was still present. These observations suggest a close relationship between naturally occurring and immune antigen–antibody systems.

Introduction

The efficiency of intravenous injections into newborn mice for the production of immunological tolerance to tissue homografts has been established. The adaptive period during which homologous tolerance can be conferred extends at least 24 hours into postnatal life in this species (1). Injection, into the embryo or newborn animal, of postmitotic foreign cells or of soluble antigens has in some instances been successful, but in others has produced only partial or temporary tolerance as estimated by homograft survival times or serological immune responses (2, 3, 4).

Depression of serological immune responses to antigens to which the experimental animal has been exposed in the tolerance-responsive period is separable from tolerance to tissue homografts conferred by the injection of whole blood or tissue cells (1, 5). It has been postulated that the perpetuation of "serological" tolerance requires persistence in the organism of the antigen concerned (6). On the other hand, according to a prevalent viewpoint, the perpetuation of

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homograft tolerance is dependent upon the maintenance of chimerism (3). A unifying concept is to be found in Burnet's theory of clonal selection (7).

The occurrence in many mammalian species of naturally occurring antibodies to a variety of antigens is widely regarded as probably representing a response to heterogenetic antigenic material encountered by the animal in the ordinary course of its existence (8, 9). Natural agglutinins for sheep and chicken red cells have been investigated in 13 inbred strain of mice by Stern and Davidsohn (10). It is the purpose of the present report to describe a study of agglutinins for chicken red cells in mice of one of these strains injected, shortly after birth, with chicken erythrocytes.

Materials and Methods

The experimental animals were the F_1 generation produced by random mating of C57L mice obtained from Jackson Memorial Laboratories, Bar Harbor. Within 1 to 6 hours of birth, certain of the newborn mice (group I) were injected intravenously with 2.5×10^7 thrice-washed chicken erythrocytes suspended in saline. This was accomplished by the insertion of a fine glass needle into the anterior facial vein. Three minutes after injection, films were made of blood obtained from the tip of the tail. The blood films were examined microscopically for chicken erythrocytes, confirming the success of injection (Fig. 1). Individual litters were kept in separate cages. The control group (group II) consisted of similarly bred F_1 litters maintained under identical conditions but receiving no injection of chicken erythrocytes in the neonatal period. All animals were bled at 6 weeks of age and the sera separated for agglutinin titration.

Agglutinin titrations were carried out in doubling dilutions of serum in 10% bovine serum albumin in saline. Each serum dilution was set up in a capillary tube with an equal volume of a 5% suspension of washed chicken erythrocytes in 10% bovine serum albumin in saline. The tubes were supported in a plasticine block at an angle of 45 degrees. After incubation for 5 minutes at 37° C and for 5 minutes at room temperature, agglutinin titers were read macroscopically.

When 16 weeks of age, the animals of group I were divided into two equal subgroups by the separation of random pairs taken from each litter. One of these subgroups (group I*a*) and the control group (group II) were injected intraperitoneally, three times at 1-week intervals, with 0.05 ml of a 50% suspension of chicken red cells. The other test subgroup (group I*b*) was not injected. Blood sera were collected from all of the animals, 1, 2, and 3 weeks after the third injection, and agglutinin titers were determined by the method described above. When the agglutination tests were repeated in saline no significant differences in titer were obtained. All chicken erythrocytes used in these experiments were obtained from one bird, a White Plymouth Rock hen of blood type B_1B_1.

PLATE I

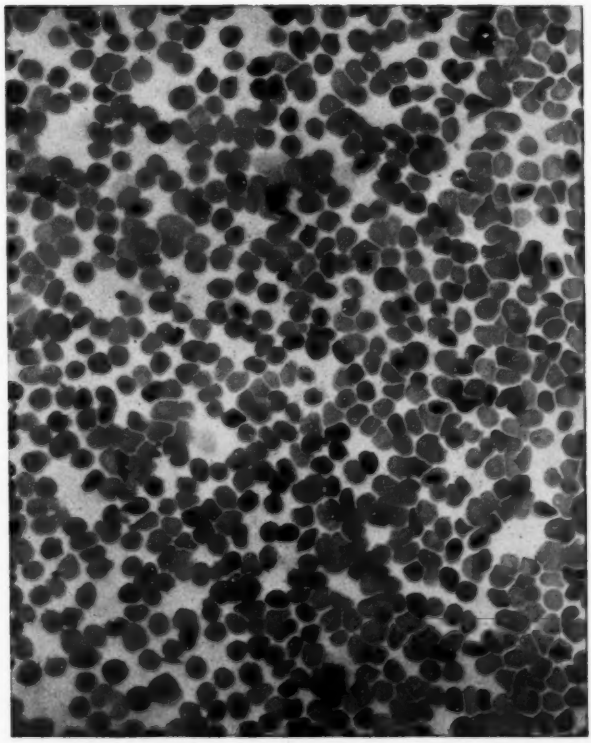

FIG. 1. Blood film from tail of newborn mouse 3 minutes after injection of chicken red cells into the anterior facial vein. Wright stain.

TABLE I

Comparison of mean $\log_2$ titers for chicken erythrocytes

	Group I	Group II	Group I*b*	Group II	Group I*a*	Group II	Group I*b*
					Chicken erythrocytes injected I.P. at 16, 17, and 18 weeks of age		
	Chicken erythrocytes injected I.V. 1–6 hours after birth	No I.V. injection	Chicken erythrocytes injected I.V. 1–6 hours after birth	No I.V. injection	Chicken erythrocytes injected I.V. 1–6 hours after birth	No I.V. injection	Chicken erythrocytes I.V. 1–6 hours after birth; no I.P. injection
Age (weeks)	6	6	16	16	21	21	21
Numbers	28	10	14	10	14	8	14
Mean $\log_2$ titers	0.71	2.70	2.43	3.30	4.07	6.38	2.64
	$p < 0.001$		$0.005 > p > 0.001$		$p < 0.001$		
Standard deviation	0.79	0.95	1.12	1.27	0.79	1.32	0.97

NOTE: I.V. intravenous, I.P. intraperitoneal.

Results

Comparison of groups I and II shows that neonatal injection of 2.5×10^7 chicken erythrocytes, while not preventing the development of natural agglutinins for chicken red cells in all animals, was associated with a significant depression of mean titer when the mice were 6 weeks old (mean $\log_2$ titer of group I 0.71, group II 2.70, $p < 0.001$). When the mice were 16 weeks old, the mean natural agglutinin titer of the injected group had risen (mean $\log_2$ titer of group I*b* 2.43), but the value was still significantly different from that of the control group (3.30, $0.005 > p > 0.001$).

In mice which had been injected with chicken cells as neonates (group I*a*) and were given multiple injections of chicken erythrocytes when 16 to 18 weeks old the agglutinin titers showed a pronounced rise (mean $\log_2$ titer at 21 weeks 4.07). Nevertheless, the fact that the antibody response of these animals was impaired is demonstrated by comparing agglutinin titers of sera obtained at the age of 21 weeks with those of the control animals (group II, mean $\log_2$ titer 6.38) challenged with the antigen and bled at the same ages ($p < 0.001$). At the conclusion of the experiment the titers of the animals in group II were still rising, while those of group I*a* were falling.

Discussion

It is Wiener's opinion that naturally occurring antibodies do not differ fundamentally from immune antibodies, probably arising in response to antigens from the environment (8). As Billingham has emphasized, the phenomenon of actively acquired tolerance is to be regarded not as the absence of an immunological response, but rather as a specific immunological response characteristic of a particular stage in the development of the immunological mechanism (11). The induction of partial tolerance in a natural antibody system, and the extension of this state into the corresponding immune system, suggest a similar relationship between antigenic stimulation and immunological response in the two systems. It may even be supposed that identical clones of immunologically competent cells are involved in both systems. At any rate, success in producing temporary partial tolerance in the test animal can be taken as evidence in favor of at least a cross reaction between chicken cell antigen(s) and the supposed natural antigen(s) should it be that the two are not chemically identical.

It has been suggested that perpetuation of a state of specific immunological tolerance depends upon the continued presence of antigen in the organism. Owen (5) has suggested that the use of erythrocytes, since they have a finite life span of intact existence, provides an antigen of limited duration. Tolerance induced by their use might therefore be expected to wane. The rise in natural heteroagglutinin titer after an initial period of depression observed in mice injected shortly after birth with the corresponding heteroantigen might be susceptible to explanation by such an hypothesis, but the impaired response to subsequent challenge by the antigen is not satisfactorily accounted for on this

basis. Favoring the view that tolerance depends upon the presence of antigen is the possibility that tolerance may be fortified by administration of antigen under some conditions (6, 12). If the appearance of antibody in the control animals is accepted as an indication of natural acquisition of antigen from the environment, the waning of tolerance in animals sharing this environment cannot be ascribed to disappearance of the antigen. On the other hand, a critical minimum amount of antigen may be assumed necessary to perpetuate a given level of tolerance. The environment may provide insufficient antigen to maintain such a quantity in the organism. An alternative assumption is that the supposed natural antigen is qualitatively incapable of contributing to the tolerant state.

The agglutinin response to intraperitoneal injection with chicken red cells in mice rendered partially tolerant by intravenous injection in the first hours of life was of a lesser magnitude than that in the control animals. Furthermore, the mean titer of the former was in decline while that of the latter was still rising. In this connection it is to be emphasized that the challenge was by multiple injections, for Owen (13) observed a possibly analagous depression of antibody response in chickens which had been injected with human blood at 1 day of age and doubly challenged at 3 months. Coincident with this depression he stated that a marked development of incomplete antibody was observed. This was not observed with the albumin and saline agglutination technics employed in the present work.

Success in producing, in chickens, immunological tolerance to turkey blood, in the face of failure to demonstrate tolerance to goose or duck blood in similar experiments, has been attributed to taxonomic diversity between donor and recipient (14, 12). This would not appear to sustain a generally applicable principle in view of the demonstration of a degree of tolerance to human red cells in chickens (13) and to chicken red cells in mice. Billingham and Brent (15) have emphasized this, recognizing that the concept of taxonomic diversity as a limiting factor in acquired immunological tolerance applies solely to tissue transplantation antigens.

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THE EFFICIENCY OF THE RESPIRATORY MUSCLES IN OBESITY[1]

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Abstract

The work done to overcome the elastic resistance and the efficiency of the respiratory muscles were determined in normal and obese subjects. The work done was no greater in the obese subjects, but the efficiency of the muscles was low. These findings suggest that the high oxygen cost of breathing in obesity is due to inefficient respiratory muscles rather than to an increased amount of work required to overcome elastic resistance. When an extrapulmonary elastic resistance was applied to the normal subjects, the compliance of the chest wall and the efficiency of the respiratory muscles fell to the level of that in the obese. This suggests that the inefficiency of the respiratory muscles of obese individuals may have been due to the reduced chest wall compliance or to the lower lung volume at which ventilation took place.

Introduction

The oxygen cost of an increase in ventilation is high in obese subjects (1, 2). Since the mechanical properties of the lungs are generally normal (2), and the compliance of the chest wall is reduced (3), the high cost of breathing has been attributed to an increased work of breathing required to overcome the elastic resistance of the chest wall (3, 4). However, the mechanical work done to overcome elastic resistance during breathing has not been measured in obese individuals. Furthermore, the possibility that the elevated oxygen cost of breathing in the obese is related to an inefficiency of the respiratory muscles has not been excluded.

The purpose of this paper is to present studies of the efficiency of the respiratory muscles and the mechanical work done to overcome elastic resistance during breathing in normal and obese individuals. In addition, an attempt has been made to elucidate the mechanism leading to the derangements observed in the obese subjects.

Methods

The subjects were seven healthy male individuals (Nos. 1–7) and one male and six female obese individuals (Nos. 8–14), each of whom weighed at least 25% more than their ideal weights* and who had no clinical evidence of cardiovascular or respiratory disease. The physical characteristics of these subjects are shown in Table I.

The subjects were studied on three separate occasions. Ventilatory function was assessed on one occasion; the compliances of the total respiratory system,

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Contribution from the Department of Medicine, the University of Manitoba School of Medicine and the Clinical Investigation Unit, Winnipeg General Hospital, Winnipeg, Manitoba.

*Ideal weights were obtained from tables published by the Metropolitan Life Insurance Company.

the lungs and chest wall, were assessed on another; and the oxygen cost and efficiency of the respiratory muscles were assessed on a third occasion.

The vital capacity and maximum breathing capacity determinations were made on a 9-liter Collins spirometer, with the valves and carbon dioxide absorber removed and a high-speed rotating drum incorporated. The maximum of at least three trials was recorded. Predicted values were determined from the data of Baldwin, Cournand, and Richards (5, 6). The distribution of inspired gas, measured by analysis of the nitrogen concentration of each breath during 7 minutes of breathing pure oxygen and of an alveolar sample at the end of the 7 minutes (7), was within normal limits in all subjects.

The compliances of the total respiratory system, the lungs and the chest wall, were determined by the body plethysmographic method which has been previously described (3). This method entails the measurement of the volume changes produced by a series of negative pressures applied around the body in random order. The relationship between volume changes and pressure was linear over a range of volume which was about two to three times the normal tidal volume. The value for compliance was calculated from this linear portion of the curve.

The work done against the total elastic resistance of the respiratory system (E. W.) at rest was calculated from knowledge of the compliance of the total respiratory system and the tidal volume. Thus,

$$\text{E. W.} = \frac{P}{2} \times V,$$

where P is the pressure change in cm H_2O, and V is the volume change in ml. Since compliance (C) = V/P and therefore $P = V/C$, the equation becomes E. W. = $V^2/2C$.

The elastic work per minute was calculated by multiplying the elastic work per breath by the respiratory frequency, while the elastic work per liter of ventilation was calculated by dividing the elastic work per minute by the minute volume.

Determination of the oxygen cost of an increased ventilation and of the efficiency of the respiratory muscles was carried out using the method of Campbell, Westlake, and Cherniack (8), as modified by Cherniack (9). Measurement of the oxygen cost entails measurement of the increases in oxygen consumption associated with increasing ventilations which have been induced by having the subject breathe through lengths of dead space. The mechanical efficiency for handling added respiratory work was calculated by measuring the increase in oxygen consumption that is associated with breathing against a known added inspiratory resistance. The subject inspired through 1-in. brass tubing under a water seal; thus he required, throughout respiration, a constant additional alveolar-spirometer pressure gradient that was relatively independent of the rate of air flow and which left expiration unimpeded. The added inspiratory pressure was measured by a water manometer and the added me-

chanical work in kg-m/minute was calculated by multiplying the added pressure in cm H_2O by the minute ventilation in liters by 10^{-2}. The extra oxygen consumption associated with the extra work load was converted to kg-m/minute, assuming a respiratory quotient of 0.8.

Results

As is seen in Table I, the compliances of the total respiratory system and its components were lower in the obese than in the normal individuals, the most marked difference being in the compliance of the chest wall. The mean chest wall compliance in the obese individuals was only 50% of that in the normal subjects.

In Table II, the rate and depth of breathing, the oxygen cost and efficiency of the respiratory muscles, and the work done to overcome elastic resistance in the normal and obese subjects are shown. It may be seen that the oxygen cost of increased ventilation was higher and the efficiency of the respiratory muscles lower in the obese subjects. Although the mean work done to overcome elastic resistance is higher in the obese, there is no statistical difference between the two groups of individuals.

In order to determine the mechanism leading to the low efficiency of the respiratory muscles in the obese subjects, it was of interest to determine the effect of a decrease in chest wall compliance on the efficiency. With this in mind, an additional elastic resistance was applied to the chest wall of the normal subjects. This took the form of a pressure vest from a G-suit which was inflated to a pressure of 15–20 cm of water at maximum expiration. Ventilatory function, lung volumes, the compliances, the oxygen cost of increased ventilation, and the efficiency of the respiratory muscles were reassessed in all of the normal subjects after application of the external resistance. Under these conditions, the mean vital capacity decreased by 530 ml (range of 230–1005 ml) and the mean maximum breathing capacity decreased by 23 l./minute (range 8–33 l./minute). The total lung capacity and functional residual capacity fell in each of the four subjects in which they are measured, the mean fall in total lung capacity being 750 ml, and that of the functional residual capacity being 935 ml.

The effect of the extrapulmonary resistance on the respiratory pattern and the respiratory compliances is shown in Table III. The tidal volume was decreased when the subject was breathing against an added extrapulmonary resistance, while the mean respiratory frequency increased from 11.9 to 16.2 per minute. In addition, the added resistance caused a fall in the compliance of the total respiratory system. This was due to a fall in chest wall compliance, no consistent variation in the lung compliance being noted.

In Table IV, the effect of the extra elastic resistance on the oxygen cost of increased ventilation, the efficiency of the respiratory muscles, and the work done to overcome elastic resistance is shown. It can be seen that the work done to overcome elastic resistance and the oxygen cost of increased ventilation

TABLE I

Physical characteristics of normal and obese subjects

Subject No.	Sex	Age (years)	Ht. (cm)	Wt. (kg)	Vital capacity		Maximum breathing capacity		Compliance (l./cm H_2O)		
					ml	% pred.	l./min	% pred.	Total	Lung	Chest wall
Normal											
1	M	21	178	77	5685	129	189	139	.088	.136	.247
2	M	21	188	79	6125	122	222	155	.119	.264	.217
3	M	18	182	68	4810	106	191	135	.061	.135	.111
4	M	20	179	75	5820	131	187	137	.141	.266	.298
5	M	24	175	70	5620	132	202	155	.099	.162	.252
6	M	21	168	68	4650	115	183	144	.129	.210	.333
7	M	35	173	82	4410	110	120	102	.103	.198	.212
Mean		23	178	76	5303	121	185	138	.106	.196	.239
Obese											
8	M	34	176	140	2970	74	55	47	.059	.140	.103
9	F	54	167	93					.038	.148	.051
10	F	48	156	103	2650	105	71	91	.059	.193	.080
11	F	70	156	87	1985	91	61	89	.070	.167	.121
12	F	34	174	123	3510	106	105	105			
13	F	55	160	88	2900	115	43	55			
14	F	42	163	168	2770	98	70	81	.069	.108	.169
Mean		48	165	115	2797	98	68	78	.059	.151	.105

TABLE II

Respiratory pattern, work, and efficiency of respiratory muscles in normal and obese subjects

Subject No.	Tidal volume (ml)	Respiratory rate (No./min)	Ventilation (l./min)	O_2 cost (ml/l.)	Efficiency (%)	Elastic mechanical work	
						kg-m/l. vent.	kg-m/min
Normal							
1	854	10.9	9.1	.77	9.5	.050	0.45
2	875	5.5	4.8	.99	12.0	.037	0.18
3	670	9.6	6.4	.49	7.9	.055	0.35
4	453	12.7	5.6	.95	7.3	.016	0.09
5	898	8.7	7.8	.89	11.0	.045	0.36
6	405	12.4	5.0	.62	9.6	.016	0.08
7	480	24.0	11.5	1.24	10.8	.023	0.26
Mean	662	11.9	7.2	.85	9.7	.035	0.25
Obese							
8	405	10.6	5.2	1.72	4.9	.029	0.15
9	820	10.6	8.7	3.46	3.3	.110	0.96
10	460	11.7	5.4	1.83	6.1	.039	0.21
11	335	15.0	5.0	1.20	5.4	.024	0.13
12	872	16.0	14.0	2.80	3.1		
13	768	17.5	13.4	3.27	3.3		
14	564	22.5	12.7	5.90	1.2	.040	0.52
Mean	602	14.3	9.2	2.88	3.9	.048	0.39

TABLE III

The effect of an extrapulmonary resistance on compliance and respiratory pattern in normal subjects

Subject No.	Compliance (l./cm H_2O)						Tidal volume		Respiratory rate		Ventilation	
	Total	% of control	Lung	% of control	Chest wall	% of control	ml	% of control	No./min	% of control	l./min	% of control
1	.036	41	.122	90	.051	19	535	63	17.6	161	9.4	104
2	.045	38	.187	71	.059	27	375	43	13.0	237	4.9	102
3	.027	44	.225	167	.032	29	514	77	11.7	122	6.0	94
4	.056	40	.197	74	.079	26	333	74	19.5	154	6.5	116
5	.049	50	.288	178	.060	24	542	60	16.0	184	8.7	112
6	.053	41	.204	97	.072	22	264	65	16.5	133	4.5	90
7	.037	36	.122	62	.053	25	347	72	19.0	79	6.6	57
Mean	.043	41	.192	105	.058	25	416	65	16.2	153	6.6	96

increased, while the efficiency of the respiratory muscles fell in each subject, the mean efficiency falling from 9.7% to 4.4%.

TABLE IV

The effect of an extrapulmonary resistance on work and efficiency of the respiratory muscles in normal subjects

Subject No.	O_2 cost		Efficiency		Elastic mechanical work			
	ml/l.	% of control	%	% of control	kg-m/l. ventilation	% of control	kg-m/l. min	% of control
1	1.40	182	7.0	74	.074	148	.68	151
2	1.92	194	4.7	39	.048	130	.21	117
3	1.05	214	4.8	61	.095	173	.68	194
4	1.15	121	3.4	47	.029	181	.20	222
5	.92	104	1.8	16	.055	122	.50	139
6	1.24	200	5.5	57	.025	156	.12	150
7	1.39	110	3.7	34	.047	204	.20	77
Mean	1.30	161	4.4	47	.052	159	.37	149

Discussion

Although there is a difference in gross age and sex between the normal and obese subjects in this study, the values obtained for the compliances, the oxygen cost of increased ventilation, and the efficiency of the respiratory muscles in the normal subjects of this series compare well with those of normal subjects of similar age and sex as the obese of this series (9). In addition, the values for the oxygen cost and the compliances of the obese subjects are comparable to those previously reported in obesity (1, 3).

The data presented suggest that the work done to overcome elastic resistance was no greater than normal in the obese individuals. This is contrary to previous views which suggested that the elastic work may be more than twice the normal value (3). However, in the latter report, the elastic work was calculated assuming a tidal volume of 1 liter. Since the tidal volumes are considerably smaller, and the calculation of elastic work is extremely sensitive to the size of the tidal volume, as well as the compliance, the previous estimation may well have been an exaggeration of the amount of work done. The high oxygen cost of increased ventilation in obese individuals therefore is not necessarily attributable to an increase in the work of overcoming elastic resistance, and may be due to an inefficiency of the respiratory muscles.

The mechanism of the inefficiency of the respiratory muscles in obese individuals is difficult to explain. In contrast to the data of Cara *et al.* (10), in which lung compliance fell, the application of an extrapulmonary resistance in the present study caused a fall in chest wall compliance but no change in lung compliance. The imposed alteration of the compliances of the "chest wall" in normal subjects, so that they resembled those seen in the obese, was associated with a fall in the efficiency of the respiratory muscles to the range found in the obese subjects. This suggests that the low efficiency found in obese individuals may be related to the altered chest wall compliance. On the other hand, the

application of the extrapulmonary resistance also reduced the functional residual capacity. Since the functional residual capacity is frequently reduced in obese individuals (11, 12), it is also possible that the inefficiency of the respiratory muscles in the obese subjects and the normal individuals with thoracic restriction may be related to the low lung volume at which they are breathing.

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NUTRITIONAL FACTORS AND THE BIOSYNTHESIS OF UBIQUINONE

THE EFFECT OF YEAST LIPIDS ON THE HEPATIC UBIQUINONE OF NORMAL AND VITAMIN A DEFICIENT RATS[1]

W. E. J. PHILLIPS

Abstract

The feeding of a ubiquinone-free diet and of diets containing ubiquinones 30 and 45 to normal and vitamin A deficient rats has been studied. Removal of the yeast-lipid component of a semipurified diet or the replacement of ubiquinone-rich vegetable oil with an oil deficient in ubiquinone invoked increases in hepatic ubiquinone levels of normal and vitamin A deficient rats. Dietary ubiquinones are not essential precursors of tissue ubiquinones in normal or vitamin A deficient rats; however, they do influence homeostatic mechanisms.

Introduction

Ubiquinone (1), a group of substituted 1,4-benzoquinones, accumulates in the liver of vitamin A deficient rats (2, 3, 4). Little information is available on the homeostatic and biosynthetic mechanisms influencing tissue levels of ubiquinone. Any study related to the biosynthesis of ubiquinone must consider two aspects, namely, the biosynthesis of the isoprenoid side chain and the origin and availability of the 1,4-benzoquinone ring. It has been demonstrated by Gloor and Wiss (5, 6) and Phillips (7) that vitamin A deficiency in the rat imposes a metabolic block between squalene and its cyclization to sterols. It is considered (7) that this effect results in the increase in hepatic ubiquinone in vitamin A deficient rats by shifting isoprenoid metabolism to isoprenologues of farnesyl rather than to condensations of farnesyl to form squalene. These studies were all related to the biosynthesis of the isoprenoid side chain and gave no information on the origin of the benzoquinone ring. All the studies reported in the bibliography (1–7) on vitamin A deficiency in the rat were with animals raised on diets containing either dried brewers' yeast or "Marmite" as the source of B vitamins. It is now recognized that yeast contains significant quantities of ubiquinone 30 (8, 9). Martius' group (10, 11) has demonstrated that ubiquinone can be formed enzymatically by a condensation between 5,6-dimethoxy-2-methyl benzoquinone and the pyrophosphate ester of the polyisoprenoid alcohol. The enzyme was localized in the mitochondria. It is important then that the influence of dietary ubiquinones or of a source of 2,3-dimethoxy-1,4-benzoquinones be understood in relation to tissue levels of ubiquinone. It is also conceivable that the increased hepatic ubiquinone in the vitamin A deficient rat results from an exogenous source of the benzoquinone ring (i.e. yeast lipid), an endogenous source or a

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combination of both. The present experiments have been designed to study the influence of diets devoid of ubiquinones or containing ubiquinones 30 and 45.

Experimental

Materials and Methods

Albino rats of the Wistar strain were obtained from the departmental stock colony. The animals were weaned at 21 days of age and placed on the appropriate experimental diet. The vitamin A deficient diets were basically similar in composition. The percentage composition was sucrose 65, vitamin-free casein 18, dried brewers' yeast 8, vegetable oil 5, and salt (U.S.P. XIV) 4. The oil contained, per 100 g, 60 mg *dl*-α-tocopheryl acetate, 1.5 mg of 2 methyl-1,4-naphthoquinone, and 8000 units of vitamin D_2. The dried brewers' yeast was incorporated into the diet as such or after continuous extraction for 36 hours with ethanol. The latter product will be called extracted yeast in the following text. Either corn oil or cottonseed oil was used as the source of the vegetable oil depending on the desired ubiquinone content of the basal diet. Vitamin A supplemented animals received orally, once per week, 0.2 ml of cottonseed oil containing 500 I.U. of vitamin A acetate.

Individual livers were bulked into samples of five livers each. Saponification, chromatography, and determination of ubiquinone have been described previously (12, 13). The ubiquinone content of the livers was calculated and expressed as ubiquinone 50.

Results

Effect of Ubiquinone 30 Content of the Diet on Hepatic Levels of Ubiquinone

Experiment 1

The ubiquinone content of the diets used in the following experiment was contributed by yeast (ubiquinone 30) or corn oil (ubiquinone 45). Vitamin A deficient diets were prepared as previously described, with the incorporation of either 8% crude yeast or 8% extracted yeast. Half of the number of animals raised on each diet received an oral vitamin A supplement. The experimental design and results are shown in Table I. The animals were maintained on the experimental diets for 46 days from weaning. The vitamin A deficient animals were at the beginning of the growth plateau stage of the deficiency syndrome and thus were not grossly deficient or moribund. The absence of vitamin A from either diet retarded growth, and regardless of the vitamin A content of the diet lipid extraction of the yeast limited the rate of growth. Vitamin A deficiency per se resulted in over a twofold accumulation of ubiquinone in the liver. The exclusion of ubiquinone 30 from the diet by solvent extraction of the yeast did not reduce the ubiquinone content of the liver; in fact both the concentration and total amount of ubiquinone in the liver was increased in both the presence and absence of vitamin A. No difference was apparent in the sterol content of the livers from animals in groups 2, 3, or 4. It appeared that the combined effect of crude yeast and vitamin A deficiency (group 1)

TABLE I

Some observations on the feeding of diets containing or devoid of ubiquinone 30

Group*	Form of yeast	Vitamin A supplement	Initial live wt. (g)	Final live wt. (g)	Liver wt. (g)	% non-sap.	Vitamin A (I.U./g liver)	Ubiquinone		Sterol (mg/g liver)
								μg/g liver	μg/ liver	
1	Crude	Nil	38	123	5.50	0.467	Not	252	1386	2.61
			39	120	4.48	0.466	detected	301	1348	2.68
2	Crude	+	39	137	7.02	0.330	123	102	716	2.03
			39	136	7.29	0.361	94	112	816	2.08
3	Extracted	Nil	39	99	4.83	0.382	Not	377	1821	2.13
			39	91	5.08	0.344	detected	345	1753	2.18
4	Extracted	+	39	118	6.64	0.330	128	138	916	1.87
			39	114	6.05	0.378	106	138	835	2.02

*Ten animals per group.

resulted in the accumulation of liver sterols. Later experiments, however, have not consistently confirmed this observation.

Experiment 2

To confirm the observations of experiment 1 that the removal of yeast lipid (ubiquinone 30) from the diet increased liver ubiquinone levels in normal and vitamin A deficient rats, a second experiment was designed. Similar basal diets using either crude yeast or extracted yeast were prepared. Half of the number of rats on each diet received the appropriate basal diet or this diet supplemented with the extracted yeast lipid. The lipid extract of the dried crude yeast was found to be 9.46%. In those diets containing the supplemental yeast lipid, amounts were added to give twice the normal levels (i.e. 19% of the weight of yeast) to exaggerate any effects. The animals were maintained on the respective diets for 51 days.

The experimental design and results are shown in Table II. Groups 1, 2, 5, and 6 are comparable to those in experiment 1. The results completely confirm the previous observation that the removal of yeast lipid by extraction evokes an increase in the levels of hepatic ubiquinone in both the normal and vitamin A deficient rat. The addition of yeast lipid (groups 3 and 4) to the diets containing crude yeast had little effect on the concentration of ubiquinone in the liver. The significant effect of feeding extracted yeast (groups 5 and 6) was abolished by the addition of the yeast lipid, the ubiquinone concentrations being restored to those observed on the feeding of crude yeast. None of the treatments had a significant effect on the concentration of liver sterols.

Effect of a Ubiquinone-free Diet on Hepatic Levels of Ubiquinone

Experiment 3

In the previous experiments, the basal diets contained significant amounts of ubiquinone 45 from the corn oil component. Only the ubiquinone 30 content of the diet was modified by extraction of the yeast. Cottonseed oil replaced the corn oil in all of the diets of experiment 3. Page *et al.* (14) have shown that corn oil contains 120–210 μg of ubiquinone 45 per g of oil while little or no ubiquinone could be detected in cottonseed oil. The experimental design and results are shown in Table III. The replacement of corn oil as the source of vegetable oil in the basal diet by cottonseed oil having a lower ubiquinone content raised the concentration of ubiquinone in control groups (2 and 4) receiving the vitamin A supplement. This is evident from a comparison of the comparable groups in experiments 1, 2, and 3. Liver ubiquinone concentration differs to some extent between experiments under similar conditions. A controlled experiment comparing various vegetable oils will be necessary to confirm the apparent effect of feeding a ubiquinone-low vegetable oil. The feeding of a ubiquinone-free diet by the removal of yeast lipid again increased the concentration of hepatic ubiquinone both in the presence and absence of supplemental vitamin A. The ubiquinone content of the diet did not influence the concentration of liver sterols.

TABLE II

The effect of yeast lipid and vitamin A deficiency on the ubiquinone content of rat liver

Group*	Form of yeast	Vitamin A supplement	Yeast lipid supplement	Initial live wt. (g)	Final live wt. (g)	Liver wt. (g)	% non-sap.	Ubiquinone		Sterol (mg/g liver)
								μg/g liver	μg/ liver	
1	Crude	Nil	Nil	31	88	4.13	0.524	356	1470	2.11
2	Crude	+	Nil	31	134	7.60	0.461	77	585	1.96
3	Crude	Nil	+	31	102	4.75	0.494	348	1653	2.68
4	Crude	+	+	31	137	7.50	0.355	99	743	2.10
5	Extracted	Nil	Nil	31	80	4.50	0.570	457	2057	2.13
6	Extracted	+	Nil	31	125	7.64	0.325	112	856	1.87
7	Extracted	Nil	+	31	97	5.60	0.430	326	1826	1.91
8	Extracted	+	+	31	130	7.80	0.355	89	694	2.02

*Five animals per group.

TABLE III

Effect of feeding a ubiquinone-free diet on the hepatic levels of ubiquinone in the rat

Group*	Form of yeast	Vitamin A supplement	Initial live wt. (g)	Final live wt. (g)	Liver wt. (g)	% non-sap.	I.U. vitamin A (g/liver)	Ubiquinone		Sterol (mg/g liver)
								μg/g liver	μg/ liver	
1	Crude	Nil	36	107	4.67	0.436	Not	260	1214	2.51
			36	99	4.49	0.567	detected	260	1167	2.77
2	Crude	+	37	119	5.28	0.346	55	130	686	2.24
			37	119	5.17	0.565	96	147	760	2.37
3	Extracted	Nil	36	65	2.79	0.553	Not	364	1016	2.53
			36	73	3.31	0.571	detected	362	1198	2.77
4	Extracted	+	36	94	4.57	0.447	63	211	964	2.27
			36	98	4.68	0.400	102	190	889	2.11

*Ten animals per group.

Discussion

Rat tissues do not contain a single ubiquinone but rather a group of isoprenologues. Dialameh and Bentley (15) found that 80 to 90% of the ubiquinone in rat liver was ubiquinone 45 and the remainder was ubiquinone 50 when animals were raised on either a ubiquinone-free diet or chow. Diplock *et al.* (16) found that rat tissues may contain up to six ubiquinones; the 50, 45, 40, and 35 isoprenologues and two other quinones. Enzymes are present in the mitochondria, which will condense the pyrophosphate ester of a polyisoprenoid alcohol and the 5,6-dimethoxy-2-methyl 1,4-benzoquinone nucleus to form the corresponding ubiquinone (10, 11). In the present experiments, the ubiquinone content of the diet was decreased, by extraction of the yeast (removal of ubiquinone 30) and by the replacement of corn oil with cottonseed oil (removal of ubiquinone 45), but no decrease was observed in the hepatic ubiquinone content of normal rats. It would appear that dietary ubiquinone as a source of the benzoquinone nucleus is not an essential precursor of tissue ubiquinones. This would further support the conclusions of Rudney and Sugimura (17) that ubiquinone 30 is not readily converted to higher isoprenologues following the observations that of the ubiquinones in rat liver mitochondria only ubiquinone 30 was labelled following an intravenous injection of C^{14}-methoxyl labelled ubiquinone 30.

The present experiments further demonstrate that the increased requirement for the benzoquinone nucleus during vitamin A deficiency is not contributed by the diet.

Although dietary ubiquinone and hepatic ubiquinone are not related in a precursor–product relationship, dietary ubiquinone does influence tissue levels. The concentration of liver ubiquinone in both normal and vitamin A deficient rats is increased by decreasing the dietary ubiquinone. It is apparent that dietary ubiquinone influences the homeostatic mechanisms controlling tissue ubiquinone levels.

Acknowledgments

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LIPID PEROXIDATION IN DEVELOPING RAT BRAIN[1]

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Abstract

The experimental results indicate that the production of thiobarbituric acid (TBA) positive material, apparently derived for the most part from polyunsaturated fatty acid peroxidation, decreases with maturation of rat brain. It appears that during maturation some factor or process is gradually introduced into, or generated within, the brain which retards the tendency of unsaturates to undergo oxidation *in situ*. This process is possibly related to the maintenance of stability in adult brain.

Introduction

Adult rat brain contains a large proportion of polyunsaturated fatty acids (PFA) which are primarily associated with the phospholipid fraction (1, 2, 3). PFA are quite susceptible to peroxidation in most animal tissues (4, 5) and this process is accelerated under conditions where natural tissue antioxidants are lowered as in vitamin E deficiency (6, 7, 8). Although peroxides are known to be toxic (9, 10), adult rat brain has been shown to contain products of lipid peroxidation, the amount of which appears only slightly affected by lack of vitamin E (7, 11). The relatively high level of these lipid breakdown products and the large quantity of PFA, the major substrate for peroxidative reactions, in rat brain has stimulated an investigation into the production, and concentration, of end products in the maturing rat brain. It has been noted previously that most of these products were formed in the microsomal-soluble supernatant fraction of the cell (7). The experiments described in the present paper demonstrates that though the total amount of end products of lipid peroxidation increases until the 10th to 15th day *post partum*, the production of these materials decreases markedly with maturation. Throughout this period of growth, there is a steady increase in the concentration of PFA.

Methods

Male rats of the Wistar strain were used throughout this study. They were maintained on a commercial fox chow diet (Toronto Elevators Limited, Toronto, Ontario) supplemented with vitamin E (Rovamix E, Hofmann-LaRoche, Montreal, Que.). Immature rats were housed with their mothers during the period prior to killing.

The term brain, as used here, means the cerebral hemispheres stripped of as much white matter as possible. All other parts of the brain were dissected away.

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The tissues were homogenized in ice-cold 0.05 *M* Tris buffer, pH 7.4. Aliquots of the homogenate were removed for testing and lipid extraction. The remainder of the homogenate was incubated under an atmosphere of air in a Dubnoff metabolic shaker for 2 hours at 37° C. At the end of this time further aliquots were removed for lipid extraction and testing.

The lipid was removed from homogenized tissue by precipitation with ice-cold 10% trichloracetic acid and by extraction of the precipitate with ethanol and petroleum ether. The pooled extracts were taken to dryness under reduced pressure and redissolved in spectroscopically pure methanol. Similar volumes of solvents were carried through the procedures to act as controls. To test for peroxidation during extraction both linoleic and linolenic acids, in amounts equivalent to that of the lipid extract, were processed in a similar manner. No changes in these acids could be detected by any of the tests noted below or by gas–liquid chromatographic analysis of the methyl esters.

Products of lipid peroxidation were determined by a thiobarbituric acid (TBA) reaction similar to that of Ottolenghi (12) and by the thiocyanate procedure described by Wilke *et al.* (5). The latter procedure was applicable to the lipid extract only. The presence of conjugated diene in the lipid extract was estimated by measuring the absorbance at 232.5 mμ in the Beckman DU spectrophotometer (13, 14).

Polyunsaturated fatty acids were determined by the lipoxidase procedure of Magee (15) modified for tissue work. Occasionally these results were checked by gas–liquid chromatographic analysis using the Beckman GC-2 gas–liquid chromatograph. The analysis checked within experimental error in all cases examined.

Results

The concentration of PFA in the cerebral hemispheres increased approximately fourfold with development (Fig. 1). This increase in concentration is not as marked as the increase in whole brain PFA (Fig. 2). The latter values are based on brain weight, the increase of which follows a sigmoidal pattern (Fig. 2). Growth rate is greatest during that period in which active myelination is taking place in white matter.

The amount of TBA-positive material produced per unit weight of PFA by lipid peroxidation (16, 17, 18) decreases with age (Fig. 3). The liberation of TBA chromogen during aerobic incubation is shown in the upper curve of Fig. 3. This production, or release, is much reduced in older rats.

Malonaldehyde is the major product of peroxidation measured by the TBA reaction (17, 19, 20). Other TBA chromogens may be present, but their absorption maxima are at wavelengths different from that of the TBA–malonaldehyde chromogen (21, 22, 23). In the experimental results noted in Figs. 3 and 4 the presence of only the TBA–malonaldehyde chromogen could be demonstrated.

Pure malonaldehyde was allowed to react with TBA to obtain a calibration

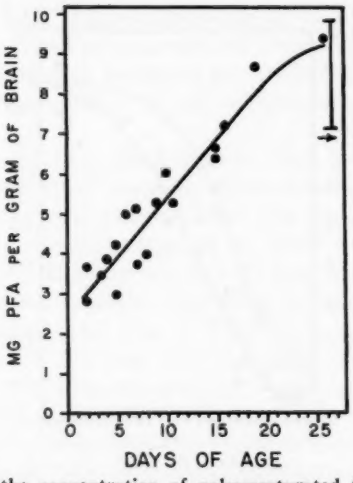


FIG. 1. Increase in the concentration of polyunsaturated fatty acids (PFA) with growth of rat cerebral hemispheres. Each point represents a minimum of eight rats. The bar in the upper right-hand corner gives the range of PFA concentrations found in adult rats.

curve which indicated that 1 μg of malonaldehyde is equivalent to 49 TBA units. Experimental results have been expressed as TBA units per mg PFA. This provides a relationship between the substrate for peroxidation, the PFA, and the end products as measured by the TBA reaction.

The values shown in Table I indicate some of the changes occurring during aerobic incubation. Immediately after excision (0 time) there are products of lipid peroxidation present in the tissue. Both the whole homogenate and the

TABLE I

Changes in rat brain homogenates after incubation in vitro*

Incubation time (minutes)	0	120
TBA values		
(*a*) Whole homogenate	101 ± 20.5†	371 ± 60
(*b*) Lipid extract	340 ± 86	355 ± 180
(*c*) Residue after lipid extraction	32	328
Thiocyanate value lipid extract	320	270
Absorption at 232.5 mμ lipid extract	0.203	0.164
PFA level (mg)	1.858	1.694

*All values are for 200 mg tissue taken from animals 15 days of age.
†Standard deviation.

lipid extract derived from it contained TBA-positive material. The thiocyanate value for the lipid extract is high indicating the presence of lipid-soluble carbonyl compounds. The absorption at 232.5 mμ is indicative of the presence of conjugated material, possibly conjugated diene hydroperoxides (13, 14, 15). After incubation the amount of malonaldehyde in the whole

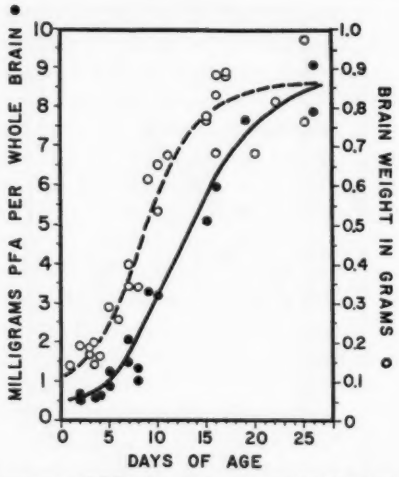


FIG. 2. The total amount of PFA in whole rat cerebral hemispheres at various stages of growth is represented by the solid line. The broken line (open circles) shows the wet weight increase with age of the same tissue.

homogenate increased, the TBA-positive material in the lipid extract remained relatively constant, carbonyls decreased slightly, and the hydroperoxide level was lowered. The concentration of PFA was slightly less after incubation.

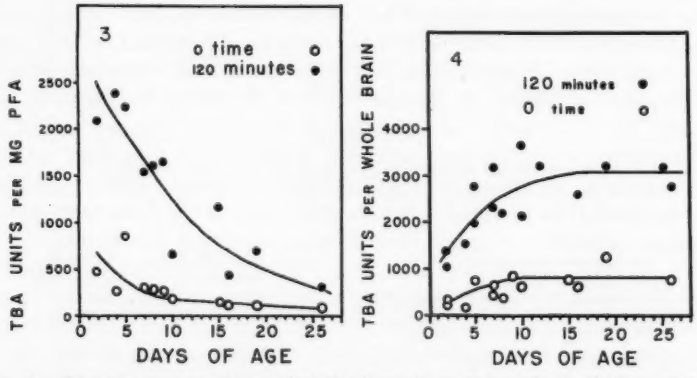


FIG. 3. The lower curve (open circles) gives the amount of peroxidation products produced per unit weight of PFA in rat brain at various stages of growth. The upper curve shows the values for the same tissues after 120 minutes of aerobic incubation.

FIG. 4. The lower curve (open circles) shows the total amount of TBA-positive material (malonaldehyde) in whole rat brain at various stages of growth. The upper curve gives the values for such material after 120 minutes of aerobic incubation.

The TBA material in the lipid extract has not been identified. Malonaldehyde could not be removed from the lipid by acid washing nor by steam distillation from an acid medium (20, 22). However, the red pigment could

not be differentiated from TBA–malonaldehyde chromogen by chromatography in 15 different solvent systems and its absorption spectrum was quite similar to that of the pigment produced by whole homogenates. Until this pigment, or pigments, is identified, it will be referred to as "bound malonaldehyde".

Figure 4 was composed from the values in Figs. 2 and 3. It seems that TBA-positive lipid breakdown products accumulate in the cerebral tissues until about the 10th day *post partum*. After this period the amount in the brain remains relatively constant although the brain continues to increase in weight and size (Fig. 2). The amount of TBA-positive chromogen produced during aerobic incubation increases in the whole brain in a similar manner to that of the endogenous material (Fig. 4, 120-minute values). The maximum level is reached about the 15th day. It appears, therefore, that peroxidation events in rat cerebral tissue remain relatively constant after the 15th day of age. Cole (4), studying peroxidation in golden hamster brain, claims that peroxide concentration in the brain of these animals reached a maximum about 10 to 16 days after birth. This is in reasonable agreement with the present work.

The results indicate that although the concentration of PFA in rat cerebral cortex increases with development, their tendency to peroxidize steadily decreases during this period. The net result is that the *in situ* level of lipid peroxidation products remains constant after the rats are 15 days old.

Discussion

The tendency of rat brain PFA to peroxidize has been shown to lessen markedly with maturation. Even when the tissues were incubated in a well-oxygenated medium the adult brain displayed less tendency to produce peroxidation products and contained a lower level of such materials. Since the actual concentration of unsaturated fatty acids continually increases during this period, it is possible that some protective process is introduced into, or initiated in, brain during its growth and development which prevents the newly formed PFA from oxidative breakdown.

Maturing brain may accumulate an antioxidant such as vitamin E. Some uncertainty concerning the function of vitamin E still exists. Some workers (24, 25, 26) claim that it functions principally as a general, natural antioxidant. The levels of this substance in brain have not been accurately determined. It would be of interest to know its distribution amongst and within the various particulate brain structures. Work of this nature has been hindered by the lack of an adequate procedure for tissue work (27). However, a recent study (28) presents a procedure which may be of great value.* Until analytical data are available the role of vitamin E, if any, in brain lipid stabilization must remain conjectural.

*NOTE ADDED IN PROOF: Since submission of this manuscript these authors have published results (Biochem. J. **79**, 91 (1961)) which indicate that adult rat brain contains a relatively low level of vitamin E.

Studies on the autoxidation of pure unsaturated fatty acids indicate that the process is initiated by oxygen but perpetuated through free radical mechanisms (29, 30, 31). The oxidations are accelerated by certain metals of which iron appears to be one of the most effective (29, 31). The over-all process in living tissue is probably much more complex although it is quite likely that the basic chemistry is similar. The regulation of oxygen tension, metallic ion balance, presence or absence of antioxidants, or reactions producing suitable redox environments could all influence the *in situ* formation of peroxides and their decomposition products.

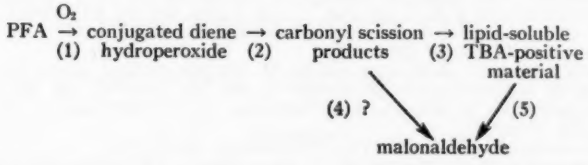
Ottolenghi (12) suggests that the mechanism in rat liver mitochondria is non-enzymatic and involves the co-oxidation of ascorbic acid and unsaturated lipid mediated by a metal ion, probably iron. Considerable work by another group (22, 32, 33, 34) has conclusively demonstrated that ascorbic acid synthesis in rat liver preparations is lowered in conditions where lipid peroxides are present. They also found (32) that metal ions (cobalt, manganese, iron) greatly influenced the ascorbic acid and peroxidation product levels in their preparations. Ascorbic acid formation takes place in the microsomal portion of the liver cell (35, 36), an area high in levels of lipid peroxidation products (7). A lipid cofactor appears to be required for the process (37). It has been suggested that the intermediary products of ascorbate synthesis (L-gulonolactone and L-xylohexulonolactone) may be more closely associated with cellular lipid reactivity than with ascorbic acid itself (33). The addition of ascorbic acid to brain homogenates does increase the amount of peroxidation products in the tissue (38). Adult rat brain cortex contains a relatively large amount of ascorbic acid (39) but whether or not brain can form ascorbic acid at any stage of growth is unknown. All ascorbate may be introduced into growing rat cortex from the blood.

Metallic ions do influence the oxidative susceptibility of whole brain homogenates from adult rats (38). Iron, vanadium, and cerium salts increased the amount of TBA-positive material of incubated brain homogenates while manganese, cobalt, copper, and thorium prevented the accumulation of oxidative products. Similar results have been obtained with the miscrosomal supernatant fraction. Metals in the brain would be associated with molecular groups. Tappel and his associates (14, 31, 40) have indicated that in liver preparations from both rat and rabbit a hematin-catalyzed oxidation of lipid occurs. The cytochromes, themselves vunerable to autoxidation (41), could act as catalysts in brain lipid oxidations.

Boiling the tissue for 10 minutes caused a rapid development of the TBA chromogen equivalent to about one hour incubation of unheated tissue. This procedure greatly lessened the ionic effect. This latter work tends to support the previous suggestion (12) of a non-enzymatic reaction mechanism. Such a system would be influenced by enzymatic reactions only so far as they supplied catalysts and cofactors.

The results presented in Table I suggest that the following reaction sequence

could be operative during aerobic incubation of cerebral tissue homogenates:



During incubation there is only a very small decrease in PFA concentration (reaction 1) but a large increase in free malonaldehyde. The initial lipid extract contained a high level of lipid-soluble TBA-positive material ("bound malonaldehyde"). After 2 hours' incubation, the lipid extract from whole homogenate contained about the same amount of this material (Table I) but the level of conjugated diene and carbonyl compounds had decreased considerably. Incubation of the residual material after lipid extraction from 0 time homogenates did not cause any further increase in the TBA value, indicating that the lipid-soluble fraction was necessary for further TBA-positive material production. After 120 minutes of incubation of the whole homogenate, followed by lipid extraction, the residual fraction had a high level of TBA chromogen in it. This tends to suggest that, during incubation, reaction 5 is operative causing an increase in free, water-soluble malonaldehyde while, concurrently, reactions 2 and 3 function to keep the level of lipid-soluble TBA-positive material relatively constant. It may be that metals such as iron and vanadium which, when added to homogenates before incubation, cause marked elevations in TBA values at 0 time, act as accelerators of reaction 5, i.e. releasing bound malonaldehyde, rather then increasing the rate of reaction 1. Preliminary studies indicate that the concentration of PFA in homogenates to which either iron or vanadium salts have been added is the same as untreated homogenates. This would support the above hypothesis. The results, therefore, strongly suggest that events occurring during aerobic incubation are mainly concerned with the breakdown of preformed peroxidic compounds and only slightly with further PFA destruction.

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EFFECTS OF ENVIRONMENTAL TEMPERATURE ON PENTYLENETETRAZOL CONVULSIONS IN CORPUS CALLOTOMIZED WHITE RATS[1]

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Abstract

Following section of the corpus callosum, white rats become more sensitive than sham-operated animals to the action of pentylenetetrazol. This is shown by an increase in the duration of convulsions and incidence of clonic, tonic, and repeated convulsions which are significantly greater in corpus callotomized rats than in control animals. In addition, in the corpus callotomized rats the effects of pentylenetetrazol appear more quickly than they do in the controls.

It was shown in the present investigation that exposure of the corpus callotomized and sham-operated rats to extremes of environmental temperature for 1-week periods resulted in an increased excitability and convulsibility of control animals. This effect was obtained following exposure to either low (4° C) or tropical (34° C) temperatures. Under similar conditions, corpus callotomized animals showed little change in convulsibility or excitability, as defined in the present study, since they had apparently reached almost a maximal state of sensitivity due to the preceding section of the commissural fibers and partial denervation of the cerebral cortical neurones; however, these animals did have a greater number of repeated convulsions when exposed to the tropical temperature, a result which may be interpreted as being due to some further increase in their sensitivity to pentylenetetrazol.

Introduction

In previous studies in which cerebral cortical neurones were partially deprived of collateral connections by aseptic section of the corpus callosum, it was found that cats and white rats became more susceptible to pentylenetetrazol-induced convulsions than control animals (1, 2, 3). During these studies an impression was gained that the excitability and convulsibility of the corpus callotomized animals were less affected by environmental changes and other types of stress than that of unoperated animals. In the present investigation, the excitability and convulsibility of corpus callotomized white rats was compared under controlled conditions with that of sham-operated controls and a stressful situation was simulated by placing the animals under conditions of high or low environmental temperature.

Experimental Procedure

In a group of 120 Sprague–Dawley rats weighing 225 ± 1.5 g, 60 animals were randomly selected. In these animals the corpus callosum was sectioned aseptically under Nembutal anesthesia. At the same time, the remaining rats were subjected to the same operative procedure without sectioning of the corpus callosum. The animals which survived the operation were paired, one

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[3]Lederle Medical Student Research Fellow.

corpus callotomized and one control animal being kept in each cage. One month after the operation the mean weights of the animals of both groups were found to be the same (351 g). At this time, subcutaneous injections of 34 mg/kg pentylenetetrazol were begun, once per week, and continued for a period of 10 weeks. Previously, it had been found that this dose produced convulsions in 50% of the rats injected (3).

The animals were maintained in a temperature-controlled room at 23° C. Between the fourth and fifth injections, the temperature of the room was lowered to 4° C and between the sixth and seventh injections it was raised to 34° C.

The frequency of occurrence and type of convulsions, the duration of the latter, the latent periods preceding excitation and convulsions, and the number of animals having repeated convulsions following any one injection were recorded, the observations being continued for 1 hour after the injection of pentylenetetrazol. Difference in convulsibility and other occurrences were tested for significance using Mainland, Herrera, and Sutcliffe's "Tables" (4). Fisher's "*T*" test (5) was employed where necessary.

Results

Description of the Responses following Injection of Pentylenetetrazol

Subconvulsive excitation.—Subconvulsive excitation by definition consisted of isolated twitches of the limbs, body, or head, and ear twitches, and blinking of the eyes. In some animals, the reaction was more severe: the animals reared on their hind limbs, flexed their front limbs, ventroflexed their heads, and tightly closed their eyes.

Clonic convulsions.—Clonic convulsions consisted of a series of rapid rhythmic limb and body twitches lasting at least 3 seconds.

Tonic convulsions.—Tonic convulsions constituted a maximal response to pentylenetetrazol. They usually began with a period of clonus followed by a tonic stiffening of all the limbs, and ended with a period of clonus. Sometimes, when very severe, these convulsions began with a tonic flexion of the front limbs, with the hind limbs extended and the head ventroflexed. This was followed by a clonic phase which began in the hind limbs and gradually spread to the rest of the body. The convulsions were preceded usually by some of the subconvulsive responses. In some of the animals more than one convulsion occurred.

For purposes of statistical evaluation, the number of animals showing any or all of the excitatory responses to pentylenetetrazol, ranging from 'subconvulsive excitation' to 'tonic convulsions', were grouped as exhibiting signs of '*general excitation*'.

Effects of Environmental Temperature Changes and Repeated Injections of Pentylenetetrazol on These Responses

The percentage of convulsions (tonic and clonic) occurring in the corpus callotomized and sham-operated control animals is shown in Fig. 1 and Table I.

TABLE I

Effects of changes in environmental temperature and repeated injections of pentylenetetrazol on the convulsibility and general excitability of corpus callotomized (CC) and control (C) rats
(Response expressed in % of animals injected)

Weekly injection	No. of animals injected		Clonic convulsions		Tonic convulsions		Total convulsions (clonic and tonic)		General excitation	
	C	CC	C	CC	C	CC	C	CC	C	CC
1	55	54	2 ← $P < 0.01$ → 35		2	4	4 ← $P < 0.01$ → 39		29 ← $P < 0.01$ → 63	
			$P < 0.05$ ↓							
2	55	54	15 ← $P < 0.01$ → 54		0	2	15 ← $P < 0.01$ → 56		31 ← $P < 0.01$ → 74	
						$P < 0.05$ ↓				
3	54	54	22 ← $P < 0.01$ → 50		9	17	31 ← $P < 0.01$ → 67		35 ← $P < 0.01$ → 78	
4	54	52	29 ← $P < 0.01$ → 63		4	4	33 ← $P < 0.01$ → 67		39 ← $P < 0.01$ → 79	
						$P < 0.05$ ↓			$P < 0.05$ ↓	
5*	54	52	35	48	9	21	44 ← $P < 0.05$ → 69		63	71
6	54	52	39	63	6	10	44 ← $P < 0.05$ → 73		50 ← $P < 0.05$ → 79	
7†	49	49	43	61	16	16	59	77	61 ← $P < 0.05$ → 86	
8	46	45	46	64	9	9	54	73	63	80
9	46	45	43	53	7	16	50	69	63	78
10	45	44	27 ← $P < 0.05$ → 50		15	25	42 ← $P < 0.05$ → 75		71	84

*Injection following 1 week's exposure to cold (4° C).
†Injection following 1 week's exposure to hot environment (34° C).

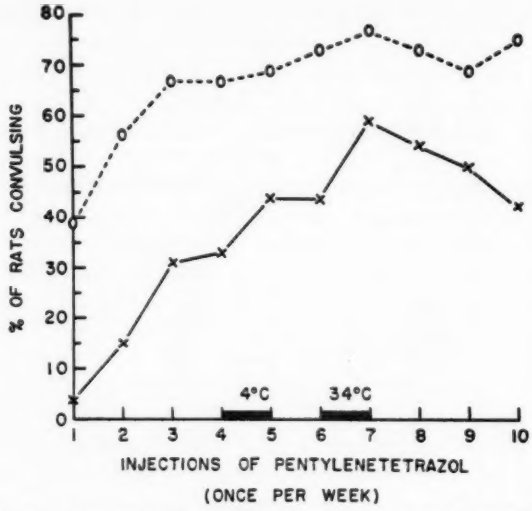

FIG. 1. Effects of changes in environmental temperature and repeated weekly injections of pentylenetetrazol on the convulsibility of corpus callotomized (– – –) and control (———) rats.

Corpus callotomized rats exhibited a greater predisposition to convulsions than the sham-operated animals following each injection; this difference was significant ($P < 0.01$–0.05) at all but the seventh, eighth, and ninth injections. During these latter injections the controls also reached a high level of convulsibility, thus masking the difference between the two groups. In both groups of animals there was a progressive increase in the number of rats convulsing during the first three injections, this increase being significant ($P < 0.05$) between the second and third injections. No further abrupt increases in convulsibility occurred in the corpus callotomized group of animals; however, the control group showed further increases in convulsibility following exposure to cold (fifth injection) and to tropical temperature (seventh injection). The convulsibility of these latter animals was 26% higher than it had been previous to application of stressful temperatures (fourth injection) and this increase was significant ($P < 0.05$). When both groups of animals were returned to normal room temperature, the convulsibility of the controls rapidly decreased towards the prestress levels while that of the corpus callotomized animals remained at a high level.

The corpus callotomized rats had, throughout the period of injections, a greater percentage of clonic convulsions than the controls, this being significant ($P < 0.01$–0.05) in the 1st, 2nd, 3rd, 4th, and 10th injections, which represent half the total number of observations. On the other hand, there was no significant difference between the percentage of tonic convulsions in the control and corpus callotomized animals. Besides the predominance of clonic convul-

sions, the corpus callotomized rats had a greater incidence of repeated convulsions than the controls at nearly all injection periods (Table II). Some of

TABLE II

Effects of changes in environmental temperature and of repeated injections of pentylenetetrazol (34 mg/kg) on the incidence of repeated convulsions in corpus callotomized (CC) and control (C) rats
(No. of animals having repeated convulsion expressed in % of the animals injected)

Weekly injection	No. of animals injected		% of animals having more than one convulsion following each injection	
	C	CC	C	CC
1	55	54	0	2
2	54	54	0	7
3	54	54	0 ← $P < 0.05$ →	11
4	54	52	2	8
5*	54	52	2	12
6	54	52	9	19
				↑
				$P<0.01$
				↓
7†	49	49	16 ← $P < 0.01$ →	51
8	46	45	9	31
9	46	45	22	20
10	45	44	22	25

*Following exposure to cold environment (4° C).
†Following exposure to hot environment (34° C).

the corpus callotomized animals had repeated convulsions even after the first injection of pentylenetetrazol, whereas no control animals had any repeated convulsions until the fifth injection. Following exposure to cold there was no great change in the number of repeated convulsions, in either group, but following exposure to a tropical environment more than half of the corpus callotomized animals had repeated, and extremely severe, convulsions; this increase in sensitivity was significant ($P < 0.01$). The controls also showed an increased tendency to repeated convulsions under these conditions; the incidence of multiple convulsions in the corpus callotomized rats was significantly greater ($P < 0.01$) than that in the controls. On subsequent injections, the corpus callotomized animals showed a decrease in repeated convulsions while the controls showed an increase.

The mean duration of first convulsions following an injection of pentylenetetrazol is presented in Table III. Corpus callotomized rats had more prolonged convulsions than the controls throughout the study; following exposure to cold, however, there was a marked (9-second) decrease in the duration of the convulsions in this group of animals. After 1 week in a normal environment the duration of convulsions increased to almost the precold exposure level, then showed a significant ($P < 0.05$) decrease in duration (14 seconds) following exposure to the tropical temperature. The duration of convulsions of the controls increased following the second and third injections, after which

TABLE III

Effects of changes in environmental temperature and repeated weekly injections of pentylenetetrazol on the duration (mean ± S.E.M.) of convulsions* in corpus callotomized (CC) and control (C) rats

Weekly injection	No. of rats convulsing		Latent period of convulsions (minutes)		Duration of convulsions (seconds)	
	C	CC	C	CC	C	CC
1	2	21	28 ± 2.5	19 ± 2.7	34 ± 4.0	68 ± 9.7
2	8	30	20 ± 3.2	16 ± 2.1	55 ± 8.6	71 ± 7.4
3	17	36	16 ± 2.2	13 ± 1.2	61 ± 5.0	72 ± 4.7
4	18	35	15 ± 2.2	15 ± 1.8	57 ± 4.4	73 ± 3.6
5†	24	36	16 ± 2.8	13 ± 1.7	57 ± 5.0	64 ± 4.0
6	24	38	11 ± 1.2	10 ± 1.1	57 ± 5.0	70 ± 3.0
7‡	29	38	11 ± 1.9	10 ± 1.5	41 ± 3.0	56 ± 3.5
8	25	33	10 ± 1.5	10 ± 1.2	55 ± 4.8	64 ± 3.7
9	23	31	9 ± 1.2	11 ± 1.6	48 ± 3.5	55 ± 3.5
10	19	33	9 ± 1.1	11 ± 1.1	53 ± 4.2	67 ± 3.0

*In the case of repeated convulsions following an injection, only the duration of the first convulsion is considered.
†Following 1 week's exposure to cold environment (4° C).
‡Following 1 week's exposure to hot environment (34° C).

it stayed practically constant until the exposure to the tropical temperature at which time there was a significant ($P < 0.05$) 16-second decrease in the convulsion time. Following the return to normal temperatures, the convulsions in both groups increased in duration as seen in the 8th and 10th injections.

In both groups of animals there was a gradual decrease in the latent period following repeated injections with pentylenetetrazol (Table III). The latencies in the corpus callotomized animals were consistently of shorter duration than in the control group except for the last two injections; however, because of wide variations in the latent periods the difference between the two groups was not significant.

Besides the greater convulsibility, as seen in Table I, the corpus callotomized rats showed a consistently greater general excitability than the controls at all times and this difference was significant ($P < 0.01$–0.05) in most injections. Following exposures to low temperatures for 1 week the controls showed a 22% ($P < 0.05$) increase in excitability; this was followed by a reduction in excitability when the animals were tested after a week at normal temperature. Exposure to tropical temperature for 1 week caused another increase in excitability in the controls and with subsequent injections carried out under conditions of normal temperature the excitability remained at a high level. On the other hand, the corpus callotomized animals showed little change in excitability throughout the series of injections, this reaching a high level and remaining there.

Discussion

As far as the section of the corpus callosum is concerned, the present study completely confirms earlier observations of Teasdall and Stavraky (1) and those of Seguin *et al.* (2, 3). They showed that the excitability, convulsibility,

and duration of convulsions and incidence of clonic and repeated convulsions are greater in corpus callotomized animals than in identically treated controls. These injections were carried out at normal room temperature and the convulsibility increased markedly during the first three or four injections, then the corpus callotomized animals became progressively depressed, while the excitability and convulsibility of the controls kept on increasing somewhat over a period of many weeks. Eventually, more control animals were convulsing at each injection period than operated ones.

The same type of experiment carried out in the present study in conjunction with exposure of the animals to extremes of environmental temperature leads to a different result: the convulsibility of the corpus callotomized rats reached a maximum and stayed there as if the exposure to abnormally hot and cold temperatures maintained their already high convulsibility; but at the same time an increased response to pentylenetetrazol was indicated by an increase in the number of repeated convulsions following each exposure to an extreme of temperature. In the control group, similar exposure to the high and low temperature had a more obvious effect on convulsibility. Following each exposure, there was a progressive and rapid increase in the number of rats convulsing which then declined as soon as the animals were returned to normal temperature.

The decrease in duration of convulsions following exposure to extremes of heat and cold which was observed in both the control and corpus callotomized animals is most likely a reflection of the increase in number of the more severe, tonic-type of convulsions which occurred following exposure to these stresses. As shown by Drake, Seguin, and Stavraky (6) tonic convulsions induced by pentylenetetrazol are of a shorter duration than the clonic ones.

It is thought that the increased susceptibility of the animals to pentylenetetrazol following exposure to extremes of environmental temperatures could be attributed to increased adrenocortical activity resulting from exposure to these stress-producing temperatures.

Exposure to cold is known to cause an increase in adrenocortical and thyroid activity (7, 8) and Selye (9) lists heat as one of the stresses which also causes increased activity of the adrenal cortex. In addition, it is generally accepted that the endocrine system exerts a definite influence on brain excitability. Woodbury and his associates (10, 11) have conducted extensive studies on the effects of chronic administrations of various adrenocortical steroids and thyroxine on brain excitability, as measured by the electroshock seizure threshold. They found that the threshold could be either lowered or elevated depending on the steroid administered, and that thyroxine also increased brain excitability. It is perhaps significant that Hellman *et al.* (12) found that exposure of man to a high environmental temperature leads to an increased secretion of the mineralocorticoid aldosterone by the adrenal cortex and that this is one of the steroids shown to have an electroshock lowering action by Woodbury (10).

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THE EFFECTS OF NO_2 AND SALTS OF NO_2 UPON ESTABLISHED CELL LINES[1]

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Abstract

The effects of several concentrations of NO_2, $NaNO_3$, and $NaNO_2$ respectively, upon strain L, mouse liver cells, and HeLa cells, were studied and a modified system designed to permit continuous exposure of cells to air pollutants is described. In NCTC medium 109 containing serum, cells tolerate concentrations of NO_2 up to 4100 p.p.m. and some may even tolerate 8600 p.p.m. Removal of the serum lowers the lethal concentration of NO_2 to less than 100 p.p.m. If the cells were covered only by a thin film of BSS (balanced salt solution) medium, a concentration of 100 p.p.m. NO_2 proved toxic within ½ hour. If, however, the NO_2 concentration was reduced to 5 or 10 p.p.m., cells survived a daily 8-hour exposure but many, if not most, of the cells were dead after several days. The presence of as little as 25 mg% $NaNO_2$ retarded proliferation. On the other hand, $NaNO_3$ was tolerated well in the three cell lines tested; HeLa cells seemed to be the most sensitive of the cell strains with respect to these salts.

Introduction

The great increase in air pollution over the past half century has aroused considerable concern as to its possible harmful effects upon animals and plants. Consequently, extensive studies have been conducted covering many aspects of this problem. Many of the detrimental pollutants found in air are now known (1, 2). They differ widely in kind, number, and quantity from one community to another, depending upon kinds of local industry, type of fuel commonly used, number of automobiles, etc.

Many investigations have been made upon various species or organisms to ascertain their reaction to air pollutants. These include nitrogen dioxide, the gas chosen for this study. NO_2, along with nitric oxide, occurs in oxidant "smog". It plays an important part in photochemical oxidation; in the presence of sunlight it dissociates into ozone and nitric oxide. Very little is known concerning repeated low-grade exposures to NO_2 and, as far as is known, nothing has been reported in regard to the effects of low concentrations on cells cultivated in vitro. Since it is only through organized study at the cellular level that we may hope to gain fundamental knowledge concerning the effects of pollutants, singly or in combination, and since we now have available numerous cloned cell types which are suitable for such studies, the following investigations were made.

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Materials and Methods

The cell lines employed consisted of strain L (NCTC Clone 929), mouse liver cells (NCTC Clone 1469), and HeLa cells.

Two different culture media were used: one, a "biological medium" containing 40% balanced salt solution, 40% horse serum, and 20% chick embryo extract (1:1); the other, NCTC No. 109 medium supplemented with 10% horse serum (3). This latter medium proved the more desirable for growth and was used exclusively in later experiments.

Replicate cell cultures were prepared as described by Pace and Aftonomos (4). At first, Carrel flasks and, later, modified T-60 flasks were used as culture vessels. To each Carrel flask, 1.0 ml of cell suspension was added; to each T-60 flask, 10.0 ml was added. In each experiment, following a 48-hour adjustment period, the cultures were divided into test and control groups.

Initial and final cell numbers were ascertained either by cell nuclei enumeration as described by Sanford *et al.* (5) or by means of a Coulter counter (6).

Results

(A) Effects of Sodium Nitrate and Sodium Nitrite

Since under ordinary circumstances nitrates or nitrites may be formed when NO_2 is passed through a water solution, the effects of the sodium salts added to the nutrient medium also were observed.

Solutions of $NaNO_3$ and $NaNO_2$ were prepared in the culture medium (NCTC No. 109 plus 10% horse serum) prior to filtration. Concentrations of 200, 100, 50, and 10 mg% were employed. Following the initial adjustment period, the culture media were changed. However, those of the test cultures were replaced with medium containing a known concentration of $NaNO_3$ or $NaNO_2$. The cultures were observed microscopically every 24 hours. Cell counts using the nuclei enumeration procedure were made every 48 hours throughout each experiment. The results are summarized in Table I.

These experiments reveal that proliferation of mouse liver cells exposed to $NaNO_3$ is not adversely affected at concentrations of 200 mg%, or lower. However, with as little as 10 mg% $NaNO_3$, there was some retardation of proliferation in one experiment (in which strain L was used) and in HeLa cells. Growth in all cell lines was reduced at all concentrations of $NaNO_2$ tested. With 100 mg% $NaNO_2$, almost complete inhibition of growth occurred in all cultures of mouse liver and HeLa cells, and in 200 mg%, these cells died within 2 to 5 days. Growth of strain L cells was also retarded considerably in 100 mg%. Little, if any, growth occurred in most cultures of strain L cells at 200 mg%. As might be expected, the lower concentrations, 10 mg% and 50 mg%, evidenced lesser effects on cell multiplication for all cell lines exposed.

(B) Exposure of Cells in "Biological Medium" to NO_2

In preliminary tests it was found that cells cultured in "biological media" could tolerate rather high concentrations of NO_2. Nitrogen dioxide was mixed

TABLE I

A comparison of the effects of various concentrations of sodium nitrate and sodium nitrite on proliferation of strain L, mouse liver, and HeLa cells

Cell type	Length of experiment in days	Initial cell No. $\times 10^3$	Concentration in mg%				
			0	10	50	100	200
			Final cell number / Initial cell number (growth ratio)				
Sodium nitrate							
Strain L	7	290	6.2	3.1	5.5	4.5	4.1
Strain L	7	320	5.2	5.2	3.4	3.1	4.2
Mouse liver	7	170	5.4	4.4	5.2	5.0	4.8
Mouse liver	7	250	5.1	4.8	7.8	5.7	6.9
HeLa	6	420	4.5	3.2	3.9	4.0	2.9
HeLa	6	440	4.6	2.6	3.3	3.5	2.9
Sodium nitrite							
Strain L	7	290	6.2	3.3	2.8	2.4	1.1
Strain L	7	330	4.9	3.3	2.5	2.0	1.2
Strain L	7	320	5.2	4.2	4.2	3.2	3.0
Mouse liver	7	170	5.4	3.4	2.4	1.8	Dead 2nd day
Mouse liver	7	250	5.1	3.5	2.9	0.8	Dead 5th day
HeLa	6	420	4.5	3.4	2.0	0.7	Dead 4th day
HeLa	6	440	4.6	2.3	0.1	Dead 4th day	Dead 2nd day

NOTE: The results are expressed as the ratio of the final cell count compared with the initial cell count. The nutrient medium was Earle's 109 plus 10% horse serum. Each figure represents the average for 10 cultures.

with 5% CO_2 in air to obtain the various desired concentrations. There was surprisingly very little change in pH except at the highest concentrations. Every 48 hours, when the nutrient medium was changed, the cultures (Carrel flasks) were "flushed" for 30 seconds with the gas mixture, or, in the case of the controls, with 5% CO_2 – air. The vessels were then stoppered and placed in the incubator for 48 hours, at which time the procedure was repeated. Immediately prior to each medium change, cultures were randomly selected for cell counts. All cultures were observed microscopically every 24 hours. The results are presented in Table II.

Proliferation was retarded in 8600 p.p.m. and 4100 p.p.m. NO_2, although not as much in the latter concentration as in the former. When exposed to 2400 p.p.m., proliferation was retarded in most of the cultures, although in three experiments with strain L there appears to be an actual stimulation. The same was true in one experiment with mouse liver cells. In still lower concentrations (1500 and 800 p.p.m.) there were no deleterious effects; in fact, there was, at times, an increase in proliferation. In one experiment with strain L and one with mouse liver cells, the synthetic medium (NCTC No. 109 plus 10% horse serum) was used. The results suggest a stimulation of growth at these concentrations when the cells were cultivated in this medium. At least there is no evidence of a retarding action.

TABLE II

The effects of high concentrations of nitrogen dioxide on growth of strain L and mouse liver cells in "biological medium" (40:40:20)

Length of experiment in days	Initial cell No. $\times 10^3$	Concentration of NO_2 in p.p.m.					
		0	8600	4100	2400	1500	800
		Final cell number / Initial cell number (growth ratio)					
		Strain L cells (NCTC No. 929)					
9	600	9.6	5.4	5.1	5.1		
8	600	7.9	3.6	5.0	8.3		
8	550	7.4	5.4	6.7	10.1		
8	2000	2.9	1.6	1.5	2.6		
8	1800	2.7	1.2	1.8	3.1		
8	1400	3.6	2.8	2.5	3.2		
12	450	7.5				7.4	8.0
12	340	3.4				5.6	3.0
10*	500	5.5				7.8	7.8
		Mouse liver cells (NCTC No. 1469)					
6	430	6.7	5.6	5.2	7.3		
6	410	12.2	4.6	4.9	11.6		
6	500	10.6	3.6	8.8	9.6		
8	450	11.0	1.6	5.5	9.8		
9*	210	8.3				9.6	8.6

NOTE: The results are expressed as the ratio of the final cell count compared with the initial cell count. Each figure represents the average for 10 cultures.
*Cells in medium NCTC No. 109 plus 10% horse serum.

The cells appeared to be "protected" from the effects of NO_2 by the various substances present in horse serum and embryo extract as well as the amino acids in NCTC No. 109, all of which undoubtedly reacted with the NO_2. The layer of nutrient medium covering the cells was sufficiently deep to require relatively considerable quantities of NO_2 before a toxic action was noticed.

(C) Direct Exposure of Cells to NO_2

Since the results of the preceding experiments indicate that there was a "detoxifying" action by actual combination of the gas with the constituents of the nutrient medium, the experimental procedure was altered. Following an initial adjustment period of 48 hours, when the cells had begun to proliferate in the flasks, the nutrient medium was replaced with balanced salt solution and the cells then exposed to the gas.

The T-60 flasks used in these experiments were modified as illustrated in Fig. 1. At the juncture with the upper glass surface and the conical base of the vessel, a small glass tube was attached. Through this tube, by means of a hypodermic needle, liquids could be added or removed aseptically. The neck of the culture vessel was modified into a standard taper, ground-glass joint which permitted an airtight seal with the glass conducting head. The gassing head consisted of a gas inlet tube surrounded by a ground-glass sleeve joint. An outlet tube through which the gas passed from the culture flask was attached at right angles to this outer sleeve.

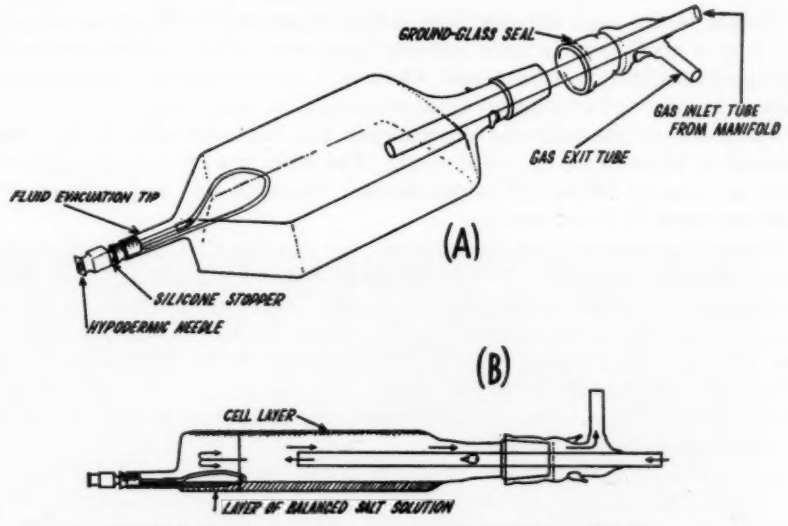

FIG. 1. Modified T-60 flask used in exposing cells to nitrogen dioxide.

The flask became filled with the gas rather rapidly (a volume equal to that of the flask passed through every minute). The cells in the culture flasks were exposed to concentrations of NO_2 ranging from 1000 p.p.m. to 5 p.p.m. The duration and frequency of the exposure varied with each concentration. Control cultures were treated identically, except that 5% CO_2 – air was used instead of the NO_2 mixtures.

Just prior to the exposure period the nutrient medium was removed from the flasks and replaced with sterile balanced salt solution (BSS). The culture vessel could then be inverted so that the cells were covered only by a very thin film of BSS. The BSS, however, remained in the flask and served to maintain a suitable humidity during the gassing interval. Following each exposure period, the cells were rinsed with the BSS.

In order to ascertain the effect of this gaseous pollutant on growth, cell numbers of test and control cultures were compared at the end of each experiment; the number of detached cells and the viability of the cells were noted at the end of each exposure period (7).

Photomicrographs were made of strain L cells exposed to 100 p.p.m. NO_2. The cells were exposed for 2 hours without interruption in the manner described above, after which the BSS was replaced with nutrient medium. Photos of these cells, as compared to cells exposed to CO_2 – air only are shown in Figs. 2A and 2B. These photos were taken about 2 hours after the cells were returned to the nutrient medium. In experiments in which higher concentrations of NO_2 were used, the cells appeared to become fixed and, with both erythrosin B and time-lapse photography, evidence indicated that all the cells were dead.

Similar results were obtained with ½-hour exposures to 100 p.p.m. NO_2, indicating a much greater toxic activity than some of the earlier results (in biological medium) had displayed. Obviously, much lower concentrations of NO_2 were indicated in order to avoid an immediate toxic effect.

Therefore, in the following experiments, the concentrations of NO_2 were reduced to 10 and 5 p.p.m., respectively. The gas mixture flowed through each flask at a rate of 100 to 125 ml per minute. The same was done with control cultures, using CO_2–air mixture.

In the first series of tests the cultures were exposed after a 48-hour adjustment period to 10 p.p.m. NO_2 for a single 8-hour interval. The cells were bathed intermittently every hour by simply inverting the flask for a few seconds. This was done to prevent drying of the thin film of solution covering the cells, although 10 ml BSS was always present in the flask as a further precaution. The results are presented in Table III. Each count is the average value for 15 cultures. The number of dead cells in the test cultures exceeded by far those in control cultures.

In the second series of tests, the cultures were exposed to NO_2 for 8 hours on each of 2 successive days. During the 16 hours between exposures, the BSS solution in the flasks was replaced with nutrient medium.

In the third series, the cells were exposed to NO_2 for 8 hours on 3 successive days with a 16-hour period between each exposure when the cells were covered with nutrient medium.

These experiments evidenced several interesting phenomena. In all the experiments, the final cell population in the experimental groups to some degree exceeded those of control groups. However, at the same time, the total number of dead cells in the experimental groups was 35 to 40 times greater. Perhaps NO_2 had a slight stimulating effect concomitant with a cytotoxic effect. This reduced the number of viable cells in the cultures exposed to 10 p.p.m. NO_2 to below that found in control cultures. The net effect, therefore, was to inhibit cell growth at this concentration and under these conditions. The degree of this inhibition increased with increased length of exposure.

In another group of experiments, the cells were exposed to 5 p.p.m. NO_2. The same procedure was employed as in the preceding; only the concentration of the pollutant was changed. The results of these experiments are also summarized in Table III.

In the first series of tests, the final cell population in the controls slightly exceeded those exposed to 5 p.p.m. NO_2. It is doubtful whether the difference was great enough to be of statistical significance. However, difference in the total number of dead cells was of considerable significance. There were about 25 times more dead cells in the test cultures receiving 5 p.p.m. NO_2 than in control cultures.

In the two series of tests in which cells were exposed continuously to 5 p.p.m. NO_2 for 8 hours for 5 days in succession, there was very little difference in the final cell population counts, but when allowance was made for the number of

PLATE I

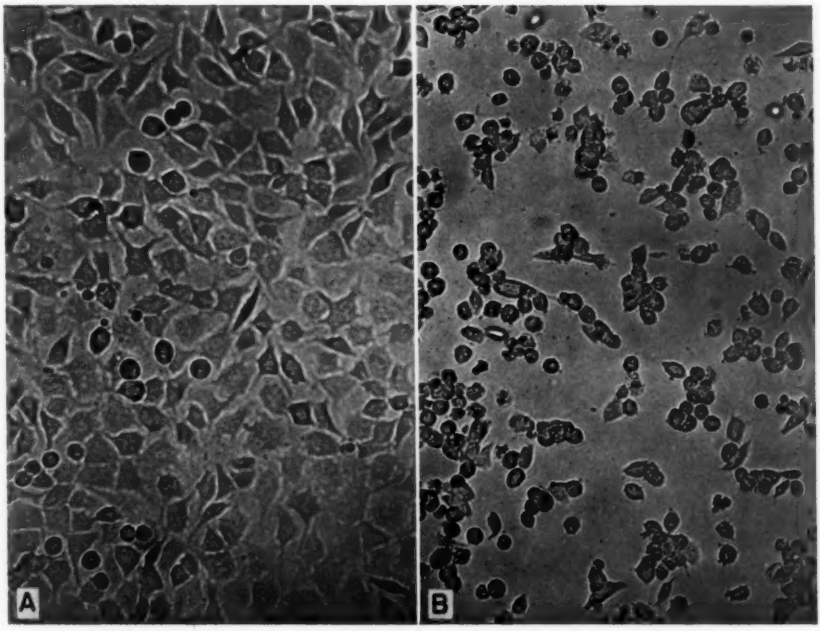

FIG. 2. Strain L cells exposed for 2 hours to (A) 5% CO_2 in air and (B) 5% CO_2 in air plus NO_2, 100 p.p.m. The photographs were taken 2 hours after the NO_2 exposed cells had been returned to the 5% CO_2–air mixture.

TABLE III

Effect of 10 p.p.m. and 5 p.p.m. NO_2, respectively, on strain L cells

Experimental conditions	Number of cells per ml	
	Controls	10 p.p.m. NO_2
1. Exposed 8 hours (1 day)		
Inoculum size	237,500	237,500
Final cell number	346,650	368,820
Number of dead cells	1,500	66,560
Number of living cells	345,150	302,260
2. Exposed 8 hours (2 days in succession)		
Inoculum size	223,650	223,650
Final cell number	503,370	718,290
Number of dead cells	10,160	402,930
Number of living cells	493,210	315,360
3. Exposed 8 hours (3 days in succession)		
Inoculum size	253,420	253,420
Final cell number	651,730	779,370
Number of dead cells	14,640	542,280
Number of living cells	637,090	237,090

Experimental conditions	Number of cells per ml	
	Controls	15 p.p.m. NO_2
1. Exposed 8 hours (2 days in succession)		
Inoculum size	246,350	246,350
Final cell number	425,870	401,620
Number of dead cells	9,120	247,150
Number of living cells	416,750	154,470
2. Exposed 8 hours (5 days in succession)		
Inoculum size	274,220	274,220
Final cell number	1,053,980	1,161,060
Number of dead cells	28,580	665,800
Number of living cells	1,025,400	495,260
3. Exposed 8 hours (5 days in succession)		
Inoculum	281,300	281,300
Final cell number	1,114,580	1,226,150
Number of dead cells	36,400	748,200
Number of living cells	1,078,180	477,950

NOTE: These cells were exposed continuously for 8 hours and then returned to control culture conditions for 16 hours before termination of experiment or re-exposure to the test condition. The numbers below represent the number of cells per ml and each is an average of 15 separate cultures.

dead cells, considerable effect was observed. There were more than 20 times the number of dead cells in cultures exposed to 5 p.p.m. NO_2 than in control cultures. Hence, the total number of viable cells in control cultures was more than twice as great as that observed in cells exposed to NO_2.

Discussion

The primary and almost sole source of NO_2 as found in polluted air is combustion of fuels. It is known to be produced in rather high concentrations in automotive exhausts, especially during acceleration (8). Its role in air pollution is not completely understood. Although it is found in concentrations of only 0.4 p.p.m. in heavy smog, Haagen-Smit believes that it speeds the oxidation of organic material in the presence of sunlight, thus abetting the production of ozone and nitro-ozonides which are considered serious hazards in the Los

Angeles type smog (9). The toxic level of NO_2 for humans is given as 20 p.p.m. The maximum allowable concentration is given as 5 p.p.m. (10).

In investigations in which gases are passed over or through media containing organic substances, especially protein and amino acids, one might expect a reaction or union to take place between some of the constituents and NO_2. However, the degree to which the medium "protected" the cells from the gas was much greater than anticipated. When administered at 48-hour intervals, 8600 p.p.m. NO_2 caused a reduction in proliferation, but 4100 p.p.m. inhibited growth only slightly. At 2400 p.p.m. NO_2 had little, if any, deleterious effect upon proliferation. When the cells were killed by the NO_2, it appeared to "fix" them. It is very doubtful whether changes in pH were directly responsible for the death of the cells except in the highest concentration (8600 p.p.m.), since no serious pH shifts were indicated. The readings were never below pH 7.0. This may be due in part to the relatively high buffering capacity of the medium.

The opinion that serum might serve as a protecting agent has been fairly well substantiated by the results observed in experiments involving direct exposure of the cells. When the serum-containing medium is temporarily removed and the cell sheet washed free of serum with BSS, much lower concentrations of NO_2 affect the cells; for example, a single 30-minute exposure to 100 p.p.m. kills the cells and apparently "fixes" them.

The modified T-60 flask (Fig. 1) proved effective in overcoming some of the difficulties which were apparent with the standard flask in studies on air pollutants. The very thin layer of BSS which covers the cells when the flasks are inverted permits more direct action of the pollutant upon the cells. Spectrophotometric determination suggests that very little reaction occurs between the pollutant gas and the BSS. This is in agreement with the fact that much lower concentrations of NO_2 were effective upon the tissue cells. We believe that this type of experimental procedure is very useful in determining the effects of various gaseous substances on tissue cells. It more nearly simulates the conditions as they exist in the lung passages.

In light of the results obtained in the direct exposure method, it may be assumed that 800, 1500, and perhaps as much as 2400 p.p.m. were ineffective because of a nullification of their action by the presence of serum. This may suggest a mechanism whereby the epithelial lining of the respiratory system is protected from more drastic effects by gases of this type.

Since nitrates and nitrites are probably produced on passing NO_2 through a medium, studies on these salts were undertaken in order to compare with those obtained with the NO_2 gas. As expected, nitrite proved to be much more toxic than nitrate. In fact, liver cells seemed to be very resistant to any ill effects that might be produced by sodium nitrate, although they were as sensitive as other cells to the action of nitrite.

This report does not include studies on cells exposed to hydrocarbons, or to hydrocarbons in the presence of NO_2, or ozone, although such investigations

are to be made. It is felt that before these complex mixtures are evaluated, it was necessary that single components be assayed, in order that proper evaluation of synergistic or antagonistic effects be made.

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THE OCCURRENCE AND DISTRIBUTION OF FREE ALKALOID SOLANIDINE IN NETTED GEM POTATOES[1]

A. ZITNAK

Abstract

Free alkaloid solanidine was detected in concentrations up to 200 p.p.m. or 33% of the total glycoalkaloid level in bitter Netted Gem potatoes. Dormant tubers of this variety are readily induced to a rapid glycoalkaloid synthesis by exposure to intense solar radiation and near-freezing temperatures, conditions frequently encountered during the potato harvest in northern areas. Under such conditions solanidine is rapidly produced in excess of amounts that could be bound as solanine, the formation of which would likely be limited by the availability of the glycosidic sugar components. The glycosidic binding of the initially synthesized solanidine proceeds at a very slow rate during the subsequent tuber rest period. Distribution studies revealed that the potato peels, representing about one seventh of the whole tuber weight, contained solanine and solanidine, respectively, at concentrations 2.5 and 6.2 times higher than the remaining tuber tissue, or approximately 30% of the total glycoalkaloid amount.

The occurrence of the free alkaloid solanidine at certain times in potato tubers of the Netted Gem variety demands a serious reconsideration of any toxicity studies which have been based on the sole use of crystalline solanine, and which have disregarded the possible existence of the free alkaloid in tuber tissues.

Introduction

The occurrence of higher than normal amounts of glycoalkaloid *solanine* in potato tubers has been repeatedly found to be coincident with a high degree of unpalatable and allegedly toxic bitterness (1, 2, 3). To date, little or no thought has been given to the possibility that the burning sensation and ill effects resulting from the consumption of bitter potatoes (2, 4, 5, 6) might be accentuated, if not directly caused, by the presence of free alkaloid *solanidine*. This alkaloid was reported to be present in senescent vines (7) and in actively growing young sprouts of certain potato varieties (8, 9), but in no instance was it shown to exist in a free state in mature potato tubers.

The problem of potato bitterness in relation to higher than normal "total solanine" concentrations has been exhaustively reviewed in numerous publications (2, 3, 7, 10, 11). Although the major effort was focused on attempts to elucidate varietal and environmental conditions leading to an abnormal solanine synthesis, the rare occurrence of free solanidine in potato sprouts and plants (8) was speculatively dismissed as incidental; it was attributed either to enzymatic hydrolysis in disrupted tissues or to hydrolysis during acid extraction of solanine (12). Accurate studies of solanidine alone are difficult because of a lack of a specific or direct determination technique applicable to plant materials. The only available procedure, which is indirect, requires a minimum solanine concentration of 150 p.p.m. and is therefore of little value in materials of low or normal solanine content (13).

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Contribution from the Department of Horticulture, Ontario Agricultural College, Guelph, Ontario.

The resolution of the problem of alleged solanine toxicity (4) is further complicated by the existence of another glycoalkaloid, chaconine, which is present in potato vines comprising one half of the total glycoalkaloid amount (14). There are, however, no reports regarding the possible presence of this compound in potato tubers.

The high incidence of exceedingly bitter tubers in the 1954 Alberta potato crop became an unprecedented aid in resolving the question of the presence or absence of solanidine in such tubers (6). Additional aid in this study was assured through the discovery of a rapid glycoalkaloid synthesis induction of dormant Netted Gem potatoes. In this case, tuber exposure to *intense solar radiation*, alternated with exposures to near-freezing temperatures during night time, was found to induce a very rapid glycoalkaloid synthesis in dormant tubers of certain potato varieties which possess an inherent ability for such a build-up (6). This finding implies that research in the field of potato glycoalkaloid build-up no longer should be restricted to the sporadic incidence of higher than normal levels of solanine during certain seasons or in widely scattered geographical regions.

This report summarizes studies on the occurrence of free alkaloid solanidine in Netted Gem potatoes exhibiting incident or experimentally induced high levels of total glycoalkaloid.

Materials and Methods

Samples of six Netted Gem potatoes, in three replications, were exposed during early dormancy to 0-, 3-, 5-, and 7-day induction periods, to initiate a rapid glycoalkaloid synthesis. Potato tissue homogenates were prepared at the end of each induction period and these were preserved at $-20°$ C and later analyzed for solanine and solanidine content.

The induction treatment consisted of a continuous day and night exposure of unprotected tubers in the field late in November 1954; at this time intense solar radiation and cool weather conditions are normal in northwest Canada. Such was actually the case; overnight temperatures below 4° C prevailed throughout the induction treatments and these were followed by intense sunshine on the first three days of exposure and partial cloudiness and haze on the fourth and fifth days. Three minimum night temperatures reached the frost levels at -3, 0, and $-2°$ C on the first, fourth, and fifth overnight periods respectively. Day temperatures were mostly below 7° C except during the fourth and fifth days when maxima of 10 and 15° C were reached.

In addition, six samples of bitter Netted Gem potatoes, submitted by complaining growers, were analyzed for solanidine presence; these samples were subjected to solanine and solanidine analysis without treatment and were considered to represent "naturally" occurring instances of bitter potatoes.

In one case a sufficient quantity of excessively bitter potatoes was secured and used for determining the distribution of free and total glycoalkaloid in the peels and in the edible tuber portion. From these potatoes, four samples of

four tubers each were selected at random for analysis. In each sample, about one seventh of tuber weight was removed as peel, and analyses for both the free and bound glycoalkaloid were carried out separately on the peels and peeled tubers respectively. Two additional samples were analyzed using whole unpeeled tubers for an accuracy comparison with the analysis of individual tuber portions.

Preparation and Extraction of Potato Material

Potato tissue homogenates were prepared from combined longitudinal one-quarter sections of each tuber. These were at first finely diced and then comminuted in a high-speed Waring Blendor for 5 minutes. In order to arrest the undesired action of oxidative enzymes in broken tissues, 0.5 ml of glacial acetic acid was added for each 100 g of material at the beginning of blending.

The preparation of potato peels for analysis demanded some deviation from this procedure and for this purpose all of the peels from any sample were finely minced after addition of 100 ml of 0.5% acetic acid.

The extraction procedure of Dabbs and Hilton, including their modification of the original colorimetric technique (15), was found highly satisfactory with and excellent recovery of added pure solanine. For example, from 10 mg of solanine (m.p. 284° C) which was added to minced potato material prior to extraction, 98.7 and 97.4% were recovered by the colorimetric and hydrolytic procedures respectively. The dependability of this extracting procedure was demonstrated earlier when a standard error of 1.5 p.p.m. was found by the authors (15).

Solanine Determination by the Hydrolysis Method

In view of the small samples used for extraction, the procedure outlined by Rooke *et al.* (13) was employed with slight modifications. The hydrolysis of a 10-ml extract aliquot (5 g potato tissue) was carried out in 30-ml graduated test tubes; these were provided with 20-cm reflux condensers made of capillary glass tubing. This was essential in order to maintain a constant liquid level during the 1-hour boiling period. The same tubes and condensers were employed when 10 ml of the hydrolyzate (2 g potato tissue) was boiled with alkaline potassium ferricyanide. The neutralization of hydrolyzate and titration of the increase in reducing power with sodium thiosulphate were performed directly in each tube, the content of which was conveniently agitated by means of a small magnetized stirring bar. The solanine equivalent of sample extracts was determined by reference to a regression line constructed by using known concentrations of solanine treated in the prescribed manner.

By the use of the described semimicro analysis, concentrations of slightly below 150 p.p.m. could be reliably determined. Although this seems to indicate little advantage over the original method it has to be borne in mind that the latter required up to 150 g of potato material for extraction.

Analytical Considerations

At the present time, the difference between the colorimetric and hydrolytic

solanine values is the only available quantitative measure of solanidine in the presence of solanine (13). The use of this indirect estimation of the free alkaloid is inevitably limited because a minimum level of 150 p.p.m. is required for the application of the hydrolytic method. This, however, is no serious problem in bitter potatoes which normally display amounts much in excess of this limit, with concentrations frequently surpassing the alleged toxicity level of 200 p.p.m. (10).

The hydrolysis method determines solanine alone by measuring the increase of reducing power on acid hydrolysis, and this increase is unaffected by the presence of free solanidine. The colorimetric method determines both solanine and solanidine, since the color reaction is based on the presence of the double bond in the steroid portion of the solanidine molecule (12), regardless of its free or bound state. The difference in values between both methods does not represent solanidine directly but its stoichiometric equivalent of solanine which is used as the reference material for calibration of the analytical technique; consequently, the difference needs to be multiplied by the factor of 0.458 to obtain the weight or concentration of solanidine alone.

The occurrence of significant amounts of free solanidine in potato tubers made it necessary to introduce two new terms which require definition. Frequent reference will be made to the *total glycoalkaloid*, which is the weight sum of solanine and solanidine; percentages of both substances can be compared and expressed accurately only in relation to their combined weight total. The "total solanine" value, which is directly determined by the colorimetric method, denotes the maximum level of solanine reached on quantitative glycosidic binding of the free solanidine. This maximum level is more explicitly described as *potential solanine*, which will be used henceforth.

Results and Discussion

1. Experimentally Induced Glycoalkaloid Synthesis

As indicated by the data in Table I, dormant Netted Gem potatoes can be readily induced to a rapid build-up of total glycoalkaloid level, a significant portion of which is present as the free alkaloid solanidine. At the end of the 7-day induction treatment, solanidine was found in a quantity equivalent to that of solanine already present; this represents 51.4% of the potential solanine level, and 32.6% of the total glycoalkaloid amount by weight.

These data indicate that most of the induced solanine found in tubers was synthesized prior to the end of the 3-day induction period; there was little change after this interval since all nine induced samples showed a strikingly narrow concentration range between 350 and 440 p.p.m. of solanine, with a calculated standard deviation of only 31 p.p.m. At the same time solanidine concentrations doubled during the sixth and seventh day of induction; this seems to indicate that the synthesis of the alkaloid alone does not become impaired as in the case of glycoside formation. The apparent resumption of solanidine synthesis in the last 2 days of exposure is attributed to a very intense

TABLE I

Solanine and solanidine concentrations in potatoes induced to glycoalkaloid synthesis

Induction period (days)	Total glycoalkaloid (p.p.m.)	Solanine		Solanidine		Potential solanine (p.p.m.)
		p.p.m.	%	p.p.m.	%	
0						55*
3	452	360	79.6	92	20.4	560
5	449	360	80.2	89	19.8	555
7	570	384	67.4	186	32.6	789

*Solanidine estimation not applicable below 150 p.p.m. level.

solar radiation and temperatures which remained below 5° C during this period. The relatively insignificant change in the amounts of solanine, once certain levels were reached, is presumably due to an exhausted supply of its component sugars. Nevertheless the fact cannot be ignored that the low temperatures during the induction treatment may have caused a shift in the glycoside–alkaloid equilibrium towards an increased rate of enzymatic hydrolysis. Probably the observation of most importance is that solanidine does exist in a free state in Netted Gem potato tubers which have a high level of total glycoalkaloid. The small differences between the 3- and 5-day treatments are considered to reflect the reduced solar radiation on the fourth and fifth day; on these days temperature maxima of 10 and 15° C were reached, and the intensity of solar radiation was reduced by atmospheric haze.

The response of Netted Gem potatoes to induction treatment by simulating conditions frequently encountered during potato harvest in northern regions supports the conclusions of earlier work (6) that intense solar radiation with coincident low temperatures are critical factors for the build-up of potato bitterness; this bitterness is an expression of high-potential solanine levels and perhaps also an expression of the presence of a free alkaloid. It is a well-known fact that most of alkaloids are very bitter in taste and usually impart a burning sensation in the mouth and throat regions.

2. Studies of Bitter Potatoes Submitted by Producers or Consumers

A slow glycosidic binding of solanidine into solanine is indicated by the data in Table II, which summarizes the results of analyses on exceedingly bitter Netted Gem potatoes received from growers and consumers over a period of 8 weeks. Considering the lapse of time between harvest, when a rapid build-up of the glycoalkaloids is expected to occur, and the dates when samples were analyzed, a decreasing trend of solanidine levels is marked. The one exception is sample F. In this particular case, potatoes were temporarily stored in a shallow pit covered with tar paper and 1 month later transferred to a regular storage. It was established that the transfer of this sample was made during conditions which would favor a resumption of glycoalkaloid synthesis; and fortunately this sample was obtained in sufficient quantity to allow a repeat analysis after a 4-week interval. The reduction of solanidine levels during this period was from 34 to 13 p.p.m., and this result is in agreement with the de-

creasing gradient (from 10.6 to 2.3%) of the remaining samples. This trend strongly indicates an eventual complete glycosidic binding of the free alkaloid, but not necessarily a change in the total glycoalkaloid content.

TABLE II

Solanine and solanidine concentrations in potatoes with incidental high glycoalkaloid levels

Date submitted	Total glycoalkaloid (p.p.m.)	Solanine		Solanidine		Potential solanine (p.p.m.)
		p.p.m.	%	p.p.m.	%	
(A) Dec. 20	499	446	89.4	53	10.6	562
(B) Jan. 8	647	581	89.7	66	10.3	726
(C) Jan. 21	323	301	93.3	22	6.7	348
(D) Jan. 25	389	375	96.3	14	3.7	406
(E) Feb. 5	499	485	97.2	14	2.8	515
(F) Jan. 25	520	486	93.5	34	6.5	560
Jan. 20	556	543	97.7	13	2.3	571

Thus it is quite evident that the existence of free solanidine in experimentally induced tubers (Table I) is not an isolated instance but of common occurrence in Netted Gem tubers showing a high level of total glycoalkaloid and the coincident bitterness. The samples investigated were obtained from widely differing environmental conditions, the potatoes having been produced in black-, brown-, and gray-wooded soil zones.

Notwithstanding the eventual, nearly complete, glycosidic binding of solanidine as solanine, the existence of the free alkaloid in potatoes, even for limited periods of time, demands a serious reconsideration or revision of solanine toxicity studies. Such studies in the past have been based on the use of crystalline solanine alone, and this might ultimately lead to erroneous and dangerous conclusions. While some of the physiological effects of solanine are reasonably well known (16), there is only limited information available regarding such properties of solanidine. Further complications in resolving the question of solanine toxicity may well arise from the plausible coexistence of solanine and chaconine in the same plant material (14). Therefore a more realistic approach must be chosen to determine adequate safety limits and maximum permissible levels of glycoalkaloid or of solanidine, whether for human consumption or livestock feeding. Such an approach should include the use of solanidine in feeding diets alone and mixed in varying proportions with solanine, as well as the direct use of bitter potatoes, or extracts thereof, containing known amounts of solanine or solanidine.

It is speculated that solanidine as an alkaloid would impart a more pronounced bitter or burning sensation than its glycoside. Dry crystalline solanine does not impart a bitter taste instantaneously as quinine or brucine will, but excites a delayed burning sensation. The perception of this sensation is greatly accentuated by the solanine being in an alcoholic or weakly acidic solution and as such it becomes quite persistent in the back of the oral cavity. Furthermore

it is speculated that the striking structural resemblance of solanidine to cholesterol shown in Fig. 1 (12) could imply an easier entry of the free alkaloid into the blood stream as compared with that of the glycoside. Even a limited entry over a period of time as inferred from cholesterol absorption studies (17) is a matter of concern since solanine poisoning is not necessarily of an acute nature. Rühl cites the case of a 2-year-old girl who died 13 days after ingesting potato berries where a post mortem showed the presence of solanine in urine (18). According to König, only a few milligrams of solanine injected into the blood stream is required to produce lethal effects, while large oral dosages reportedly did not have any marked effects (16). The latter fact seems to be associated with an irritation of the gastrointestinal mucosa which induces vomiting and thus removal of much of the solanine before it can affect the nervous system. This may not be the case with solanidine, which could be more deleterious because of its hypothetical mobility in the organism. The probability of solanidine formation within the digestive tract through acid hydrolysis of solanine seems limited. According to Prokoschev, no solanine hydrolysis takes place over a period of 8 days in 2% acid solution (19).

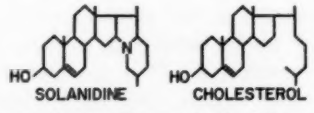

FIG. 1. Structural resemblance of solanidine to cholesterol.

It was found earlier by Smerha that sprouting potatoes had been lethal to rats, while the same potatoes, after the removal of peels, had no toxic effects (20). This also seems to implicate solanidine, since the alkaloid is freed by the action of a hydrolytic enzyme which was found in the potato sprouts by Petrochenko (21).

3. Distribution and Concentration of Solanine and Solanidine in Peels and Peeled Potatoes of High Glycoalkaloid Content

Once positive evidence was secured as to the existence of free solanidine in bitter potatoes, an attempt was made to determine the distribution of both glycoalkaloid components within the tuber; at the same time information was sought regarding the amounts of these substances that are removed by peeling. The mean values of the analyses are presented in Table III.

The potato peels contained solanidine in amounts equal to those in the peeled tuber although the peels represented only one seventh of the whole tuber weight. Thus the solanidine concentration in peels is about 6.2 times that of the remaining tuber tissue and the synthesis of alkaloid in this region is strongly indicated. At the same time the concentration of solanine in the peels was found to be only 2.5 times higher than that of the peeled tubers. Thus peeling of bitter potatoes removes about one third of the total glycoalkaloid, one half of which is likely present in a free state. In spite of the removal of these quanti-

TABLE III

Total glycoalkaloid distribution in bitter Netted Gem potatoes

Tuber portion	% of tuber weight	Total glycoalkaloid		Solanine		Solanidine		Potential solanine (p.p.m.)
		p.p.m.	%	p.p.m.	%	p.p.m.	%	
		Percentage distribution						
Peels	13.9	162	31.2	140	26.9	22	4.2	187
Peeled	86.1	358	68.8	336	64.7	22	4.2	383
Sum	100.0	520	100.0	476	91.6	44	8.4	570
Whole	100.0	509		466	91.6	43	8.4	559
		Concentration						
Peels		1161		1006		155		1344
Peeled		416		391		25		445
Whole		509		466		43		559

ties, the residual glycoalkaloid is still too high to render bitter potatoes more palatable or safe for food consumption.

In view of the fact that solanidine can be produced in excess of the amount that may be bound as solanine, it is imperative to elucidate more accurately the pharmacological properties of the alkaloid alone. Further occurrences of the free alkaloid are quite likely, since high solanine levels were reported for numerous other potato varieties (6). It is speculated that the alkaloid could enter the blood stream more readily than its glycoside. If this is so, the ill effects and some lethal cases attributed to consumption of high solanine potatoes would have been more likely cases of solanidine rather than of solanine poisoning.

Acknowledgments

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PHOSPHORUS AND LACTIC ACID METABOLISM IN THE HYPOTHERMIC RAT[1, 2]

JOHN R. BEATON AND T. ORME

Abstract

Further observations on the increase in blood levels of inorganic phosphorus and lactic acid in hypothermic rats are reported. Progressive increase in blood inorganic phosphorus levels with continued cooling to a rectal temperature of 5° C appears to be a consequence of catabolism of organic, non-lipid phosphorus. With continued cooling, blood lactic acid is progressively increased; liver lactic acid increases initially, returning, at 5° C, to control values; muscle lactic acid increases initially and thereafter remains relatively constant. Progressive increases in blood levels of inorganic phosphorus and lactic acid with continued cooling appear to be related to the depth, rather than duration, of hypothermia. Rewarming hypothermic (15° C) rats to 37° C rectal temperature with artificial ventilation reduces lactic acid levels of blood, liver, and muscle to less than control values. Rewarming alone was found to reduce blood lactic acid levels to less than control values. Elevation of blood lactic acid levels consequent upon hypothermia was prevented by provision of oxygen during cooling but not by pentobarbital anesthesia. These observations are consistent with the hypothesis that, in the hypothermic rat, the available oxygen is insufficient to meet even the reduced metabolic requirements.

Introduction

In recent communications from this laboratory (1, 2) we have reported increased blood levels of inorganic phosphorus and lactic acid in fasted rats cooled to rectal temperatures of 15° C. Results presented in these communications suggested that the increase in blood inorganic phosphorus is probably the result of breakdown of organic phosphorus compounds rather than a simple mobilization of inorganic phosphorus from tissues such as the liver. The results did not permit identification of the fraction of organic phosphorus catabolized in the hypothermic rat. Our previous reports (1, 2) suggested that the increase in blood lactic acid in hypothermic rats is due to increased formation of lactic acid rather than to decreased utilization. No measurements of lactic acid were made other than in blood. The experiments reported here were undertaken to investigate blood phosphorus fractions, blood and tissue lactic acid levels, and alterations in phosphorus and lactic acid levels with progressive cooling of the rat. These experiments were designed to investigate further the hypothesis that, in the hypothermic rat, available oxygen is insufficient to meet even the reduced metabolic requirements and, therefore, a relative hypoxia exists in the hypothermic rat.

Methods

In all experiments, adult albino rats of the Wistar strain, weighing 200–250 g and maintained on fox chow and water ad libitum, were used. Eighteen hours

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before experiment, food was withdrawn to eliminate its variable effect on metabolite levels. Cooling was carried out by placing unanesthetized animals in individual, cylindrical screen cages under ice until the desired rectal temperature was attained. Blood samples were withdrawn by syringe from the exposed heart (right ventricle) following intraperitoneal injection of sodium pentobarbital in 0.85% aqueous sodium chloride at a level of 10 mg/100 g body weight to effect rapid anesthesia. In one experiment in which prolonged anesthesia was desired, sodium pentobarbital was injected intraperitoneally at a level of 3.5 mg/100 g body weight. For tissue lactic acid determinations, approximately 500 mg of tissue (liver and gluteus maximus muscle) was rapidly excised, weighed, and homogenized in 10 ml of cold 10% trichloracetic acid with the use of a homogenizing tube and teflon pestle immersed in ice water. Determinations of inorganic, total acid-soluble, and lipid phosphorus were made by the procedure of Fiske and Subbarow (3) in tissue and whole blood. Organic, non-lipid phosphorus was calculated, by difference, from the results of acid-soluble and inorganic phosphorus assays. Lactic acid in tissues and whole blood was determined by the method of Barker and Summerson (4). In one experiment, fine copper–constantan thermocouples were inserted in liver and gluteus maximus through small incisions made in the peritoneum and overlying tissues respectively. The thermocouples were connected to a calibrated Cambridge Temperature Meter and liver and muscle temperatures were measured during cooling. In all experiments, care was taken to avoid increasing the activity of the animals since this could alter metabolite levels.

Experimental

Blood Phosphorus Levels with Progressive Cooling

Forty rats were divided into five groups of eight rats each. One group was sacrificed and served as a control. The remaining four groups were cooled to rectal temperatures of 29, 23, 15, and 5° C, respectively, and were sacrificed. Two animals of each group were used on each day to eliminate differences due to time or analytical technique.

Blood and Tissue Lactic Acid Levels with Progressive Cooling

Forty rats were divided into five groups of eight rats each and were treated as in the preceding experiment, two animals of each group (37, 29, 23, 15, and 5° C rectal temperature) being used on each day. In addition, one group of 12 rats was cooled to a rectal temperature of 15° C and then rewarmed for 15 minutes to 37° C accompanied by artificial ventilation with an oxygen:carbon dioxide mixture (95%:5%) as previously described (5). This latter group was studied to investigate the extent of return to normal of lactic acid levels on rewarming.

The Effect of Sustained Hypothermia on Blood Levels of Lactic Acid and Inorganic Phosphorus

Thirty-two rats were divided into four groups of eight rats each. One group

was sacrificed and served as a control. One group was cooled to a rectal temperature of 22° C and one group to a rectal temperature of 13° C. The fourth group was cooled to 22° C and maintained at this temperature by removal from the ice for a period equivalent to the time required to cool rats further to 13° C. Blood lactic acid and inorganic phosphorus levels were determined.

Effects of Rewarming, Oxygen, and Pentobarbital Anesthesia on Blood Lactic Acid Levels in Hypothermia

Seventy-two rats were divided into groups of eight rats each. Five groups were treated as follows: one control group; one hypothermic (15° C) group; one hypothermic (15° C) group rewarmed for 15 minutes in a 40° C water bath to a rectal temperature of 37° C; one control group placed in a container through which an oxygen:carbon dioxide (95%:5%) mixture was passed for 30 minutes; one group placed under ice in the oxygen:carbon-dioxide-flow container and cooled for 30 minutes to a rectal temperature of 15° C. Four groups were anesthetized with intraperitoneal sodium pentobarbital (3.5 mg/100 g) and were treated as follows: one control group sacrificed 25 minutes after injection of anesthetic; one group cooled under ice for 25 minutes to a rectal temperature of 15° C; one control group placed in the oxygen:carbon-dioxide-flow container for 25 minutes; one group placed under ice in the oxygen:carbon-dioxide-flow container and cooled for 25 minutes to a rectal temperature of 15° C.

Relationship of Muscle and Liver Temperatures in Hypothermic Rats

Since lactic acid levels may represent a net effect of muscle production and liver catabolism, and since such processes are temperature-dependent to some degree, an experiment was carried out to measure temperatures of gluteus maximus and liver at predetermined rectal temperatures. Six rats were anesthetized with intraperitoneal sodium pentobarbital (3.5 mg/100 g body weight) and were placed under crushed ice. Temperatures of gluteus maximus of the left leg and of the liver were measured at rectal temperatures of exactly 35, 30, 25, 20, and 15° C as cooling progressed.

Results

The results of blood phosphorus determinations are shown in Fig. 1, each point representing the mean value ± standard error of the mean for eight rats. It is evident that with progressive cooling, inorganic phosphorus increases and organic, non-lipid phosphorus decreases. Cooling was without significant effect upon acid-soluble phosphorus, as previously reported (1), or upon lipid phosphorus levels.

The results of blood and tissue lactic acid determinations with progressive cooling are shown in Fig. 2, each point representing the mean value ± standard error of the mean for eight rats. The results confirm our observation (1, 2) of an increase in blood lactic acid in hypothermia and demonstrate that this

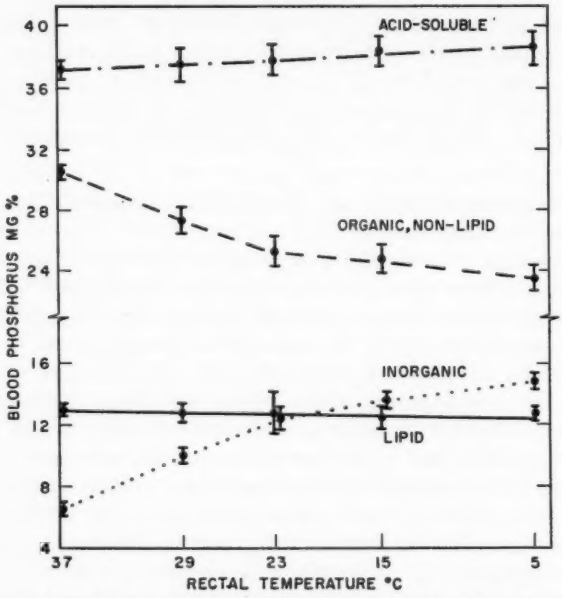

FIG. 1. Blood levels of inorganic, lipid, acid-soluble, and organic and non-lipid phosphorus in hypothermic rats. Each point represents the mean ± standard error of the mean for eight rats.

increase is progressive to a rectal temperature of about 15° C ($P < 0.001$). As cooling proceeds, muscle lactic acid levels increased to an apparent maximum at a rectal temperature of about 23° C ($P < 0.05$) and remained relatively constant thereafter. Liver lactic acid levels increased initially until a rectal temperature of 23° C was attained ($P < 0.01$) and decreased thereafter to the control level. In the rats cooled to a rectal temperature of 15° C and rewarmed to 37° C with artificial ventilation, lactic acid levels were 36 ± 3.0, 19 ± 1.7, and 28 ± 1.9 mg% in blood, liver, and muscle respectively. Thus, rewarming to a rectal temperature of 37° C with artificial ventilation, using an oxygen:carbon dioxide mixture (95%:5%) for a 15-minute period, restored elevated lactic acid levels to significantly less than initial control values, $P < 0.02$, < 0.001, and < 0.001 for blood, liver, and muscle respectively.

The effect of sustained hypothermia on blood levels of inorganic phosphorus and lactic acid is shown in Table I. Again, progressive increases in blood levels of inorganic phosphorus and lactic acid were observed with continued cooling. From the values obtained for the sustained hypothermic group, it is apparent that further increases in blood inorganic phosphorus levels with progressive cooling are related to the depth of hypothermia rather than to its duration. Blood lactic acid levels returned to less than control values in the sustained hypothermic group, which is opposite to what would be expected if duration

TABLE I

Effect of "sustained hypothermia" on blood levels of inorganic phosphorus and lactic acid (Results expressed as mean ± standard error of the mean for eight rats)

	Control (1)	Hypothermic (2)	Hypothermic (3)	Hypothermic (4)	Probability, *P*
Final rectal temperature, °C	36±0.01	22±0.49	22±0.49	13±0.49	—
Duration of cooling, minutes	—	22	44	44	—
Blood inorganic phosphorus, mg%	6.2±0.072	9.2±0.34	8.8±0.28	11.8±0.72	(1) vs. (2) < 0.001 (1) vs. (4) < 0.001 (2) vs. (4) < 0.01
Blood lactic acid, mg%	37±2.9	53±2.9	21±1.9	67±4.0	(1) vs. (2) < 0.01 (1) vs. (3) < 0.001 (2) vs. (3) < 0.001 (2) vs. (4) < 0.02

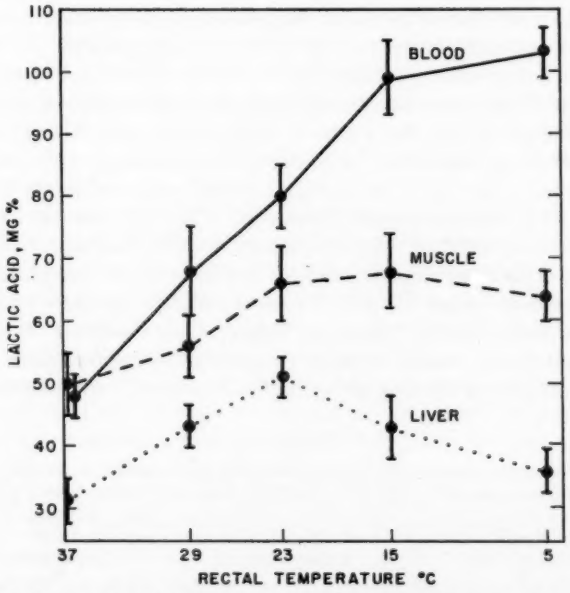

FIG. 2. Lactic acid levels in blood, liver, and muscle (gluteus maximus) of hypothermic rats. Each point represents the mean ± standard error of the mean for eight rats.

of hypothermia were the factor causing elevated blood levels of lactic acid.

The effects on blood lactic acid levels, in hypothermic rats, of rewarming, of pentobarbital anesthesia, and of oxygen are shown by the data in Table II.

TABLE II

Effects of rewarming, oxygen, and pentobarbital anesthesia on blood lactic acid levels in hypothermic rats
(Results expressed as mean ± standard error of the mean for eight rats)

Group	Experimental treatment*	Blood lactic acid, mg%	Probability, *P*
1	Control	30±2.1	—
2	Hypothermic	69±4.5	vs. 1 < 0.001
3	Hypothermic, rewarmed	22±2.1	vs. 1 < 0.02 vs. 2 < 0.001
4	Control, ventilated with oxygen	12±1.4	vs. 1 < 0.001
5	Hypothermic, ventilated with oxygen	34±1.5	vs. 2 < 0.001
6	Control, anesthetized	13±1.4	vs. 1 < 0.001
7	Hypothermic, anesthetized	30±2.1	vs. 6 < 0.001 vs. 2 < 0.001
8	Control, anesthetized, ventilated with oxygen	8±0.50	vs. 6 < 0.01
9	Hypothermic, anesthetized, ventilated with oxygen	6±0.70	vs. 6 < 0.001 vs. 7 < 0.001

*See text for details of experimental treatment.

It is apparent that rewarming alone, in the absence of artificial ventilation, lowered blood lactic acid levels of hypothermic rats to a mean significantly less than that of control animals. Pentobarbital anesthesia for a period of 25 minutes lowered blood lactic acid levels in both control and hypothermic rats but did not prevent the elevation of blood lactic acid consequent upon hypothermia. Continuous ventilation with the oxygen:carbon dioxide mixture during cooling prevented elevations in blood lactic acid in both anesthetized and unanesthetized animals and, indeed, in ventilated, anesthetized, hypothermic animals a significant lowering of blood lactic acid levels was observed. Ventilation with oxygen:carbon dioxide for 25 or 30 minutes lowered blood lactic acid levels in control animals whether anesthetized or unanesthetized.

In Table III, temperatures of muscle and liver tissue are recorded over the rectal temperature range 35°–15° C during cooling. At each rectal temperature, and in each animal, the liver temperature exceeded that of muscle. Although somewhat lower, muscle temperatures approximately paralleled liver temperatures as cooling progressed to a rectal temperature of 25° C.

TABLE III

Muscle (gluteus maximus) and liver temperatures during cooling
(Results expressed as mean ± standard error of the mean for six rats)

Rectal temperature, °C	Muscle temperature, °C	Liver temperature,* °C
35	32.9±0.40	33.5±0.25
30	27.3±0.22	28.6±0.15
25	23.1±0.58	25.3±0.33
20	16.8±0.82	21.1±0.31
15	12.9±0.54	18.7±0.66

NOTE: Muscle and liver temperatures were measured at predetermined rectal temperatures.
*For each animal, and at each rectal temperature, the temperature of liver exceeded that of muscle.

Below 25° C rectal temperature, muscle tissue cooled to a greater extent than did liver tissue.

Discussion

It would appear that the increased inorganic phosphorus level of whole blood in hypothermic rats arises from catabolism of the organic, non-lipid fraction which includes hexose phosphates, adenosine phosphates, and phosphocreatine. In a previous communication (2) it was shown that mobilization of inorganic phosphorus from tissue, such as liver, to blood is not a factor in the elevated blood level of inorganic phosphorus. It is of interest that Brock (6) has reported increases in inorganic phosphorus of plasma and erythrocytes in hibernating hamsters attributed to catabolism of phospholipids and of adenosine triphosphate and hexose phosphates respectively. Although in our studies we did not differentiate between plasma and erythrocyte inorganic phosphorus, it appears that catabolism of phospholipid is not a factor in the elevated blood level of inorganic phosphorus observed in the hypothermic rat. In our animals under these experimental conditions, it is apparent that further increases in blood inorganic phosphorus levels with progressive cooling are related to the depth of hypothermia rather than its duration (Table I). The importance of phosphorus compounds in carbohydrate, and thus lactate, metabolism is well-established and, as pointed out by Kaplan (7), an accumulation of inorganic phosphorus accelerates metabolism of carbohydrate and is unfavorable to glycogen synthesis. We have shown (2) that liver glycogen levels are very low in our fasted rats and are not a factor in altered blood carbohydrate levels.

Time and temperature relationships in lactic acid levels of blood, liver, and muscle with progressive cooling are of considerable interest. The plateauing of muscle lactic acid levels at a rectal temperature of about 23° C may indicate marked reduction in, or cessation of, metabolic production by muscle. The liver at and below this rectal temperature may lower its lactic acid level by maintaining metabolic activity since it has a significantly higher temperature than does muscle, i.e. liver maintains the ability to catabolize lactic acid at a greater rate than it is produced by muscle at this rectal temperature. Data obtained from temperature measurements (Table III) support this tentative conclusion. The continued rise in blood lactic acid levels to a rectal temperature of about 15° C probably reflects, in part at least, the reduced blood circulation due in large part to the reduction of cardiac activity in hypothermic rats (5). In support of the hypothesis that the hypothermic rat maintains ability to catabolize lactic acid during cooling, we reported previously (2) that injected lactic acid is cleared from the blood at least as rapidly in hypothermic rats as in normothermic rats. The present experiments indicate also that the continued increase in blood lactic acid with progressive cooling is due to the depth, and not duration, of hypothermia. Indeed, sustained hypothermia at 22° C results in a decreased lactic acid level in blood.

It is noteworthy that although prolonged pentobarbital anesthesia lowered lactic acid levels in blood of both control and hypothermic rats, the increase consequent upon induction of hypothermia was not prevented. In other groups in which pentobarbital anesthesia was induced just prior to withdrawal of blood samples, it might be argued that differences in blood lactic acid levels between control and hypothermic rats might have been due to differences in rate of absorption of the anesthetic. The present experiment appears to eliminate anesthesia as a contributing factor in the lactic acid increase. It was of interest to note that, with anesthetized rats, cooling time was reduced somewhat from that required for unanesthetized rats and, further, gross activity including shivering was eliminated. It would appear, therefore, that lactic acid increases in hypothermic rats are not simply explained by increased muscle activity such as by severe shivering and body movement.

In previous communications (1, 2), we have suggested that in the hypothermic rat a relative anoxia exists; that is, available oxygen is insufficient to meet the reduced metabolic activity as a consequence of the reduced respiratory and cardiac activities. This hypothesis was tested in the present study by providing an oxygen:carbon dioxide mixture (95%:5%) during cooling. Since, in both anesthetized and unanesthetized rats, this treatment prevented the elevation of blood lactic acid consequent upon hypothermia, support is provided for the hypothesis of a relative anoxia in hypothermic rats which can be overcome by provision of a high oxygen concentration mixture. Henneman *et al.* (8) noted that during hypothermia in man, with anesthesia and hyperventilation induced, there was a decrease in blood lactic acid level. Our results with provision of oxygen are in agreement with their findings and stress the importance of clearly defining experimental conditions when describing metabolic alterations of hypothermia, e.g. fasting, anesthesia, method of cooling, rate and duration of cooling, and ventilation with high concentration of oxygen. With regard to the apparent hypoxia in hypothermic rats, it is of interest that Bullard *et al.* (9) have reported that mammalian species which hibernate can survive more severe hypoxia than can non-hibernating species. In our studies in this laboratory, we have observed that hamsters, which are hibernators, can be readily cooled to rectal temperatures of 0°–3° C and revived in normal condition. Rats require some form of pretreatment in order to survive such cooling (5). It is quite possible that the difference between hamsters and rats in survival after acute hypothermia is related to a difference in susceptibility to hypoxia.

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α-REDUCTION OF THE 20-CARBONYL GROUP IN C-21 STEROIDS BY RHODOTORULA LONGISSIMA[1]

VIOLET M. CHANG AND D. R. IDLER

Abstract

The microbiological synthesis of several 20α-dihydro reference steroids is described. The influence of various substituent groups on α-reduction of the C-20 carbonyl group by a yeast, *Rhodotorula longissima*, has been investigated for compounds related to progesterone and corticosterone. Reduction at C-20 is enhanced by the presence of a 17α-hydroxyl group, but retarded by the presence of hydroxyl groups at C-11 and C-21. In those instances where 20α-conversion proceeds at a slow rate, oxygenation at C-11 appears to be essential for the preservation of the Δ^4-3-ketone structure.

Introduction

With the demonstration that reduction of the carbonyl at C-20 is a major pathway in corticosteroid metabolism (1–5), and especially since the isolation of 17α,20β-dihydroxy-Δ^4-pregnen-3-one from the plasma of postspawned female sockeye salmon (6), particular attention has been directed in this laboratory to the C-20-hydroxyl analogues of several adrenocortical steroid hormones.

The various enzymic transformations of the steroid molecule by microorganisms has been the subject of several reviews (7–9). In many cases the direct one-step microbial transformation has proved to be a tool equal to or superior to conventional chemical approaches which often entail many difficult steps and result in poor yields. One such case in point is the preparation of 20α-hydroxysteroids. The reduction of the C-20 ketone by various chemical methods yields the β-oriented 20-hydroxy compound as the major product (10–12). Recently, microbiological stereospecific conversions of Reichstein's substance S, cortisone, and prednisone to the corresponding 20α-hydroxysteroids were reported (13). A similar reduction of 17α-hydroxyprogesterone, in this laboratory, produced the 20α-dihydro-epimer in surprisingly high yield (6). It seemed desirable, therefore, to apply this facile method to the preparation of 20α-hydroxy epimers of various known adrenocorticoids. This paper is a report of the effects of common substituent groups in the steroid molecule on the rate of C-20 reduction and retention of the Δ^4-3-ketone entity.*

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*A side reaction which occurs during incubation involves the reduction of the 3-carbonyl group (13) with subsequent loss of ultraviolet light absorbency. Therefore, ring A stability, as measured by ultraviolet light absorption, will be used in this paper to denote the preservation of the Δ^4-3-ketone structure.

Methods

The yeast used throughout this investigation was *Rhodotorula longissima*, strain Schering OFV No. 2.* The reactions were carried out in the Edamin–dextrose–cornsteep liquor medium described by Carvajal *et al.* (13). Freshly grown 2-day-old cells were used to inoculate the reaction medium and incubation was at 25° C on a rotary shaker.

The rate of steroid conversion was followed readily by a quantitative paper-chromatogram analysis of aliquots at various intervals. Periodic samples were removed from the incubation mixture and extracted three times with 2.5 volumes of distilled methylene chloride. In a typical separation of the reaction products, an aliquot of the methylene chloride extract containing 50–100 μg steroid was transferred to a titerlenmeyer and evaporated to dryness at 59° C under a stream of nitrogen. The walls of the flask were rinsed into the well with methylene chloride and again evaporated under nitrogen. The residue, dissolved in a small amount of methylene chloride:methanol (1:1) was applied to a 1-cm strip of specially washed Whatman No. 1 filter paper (6). In all cases the papers were equilibrated overnight and developed in a descending solvent system at 20° C. Each paper was dried in air and scanned with ultraviolet light. An area containing ultraviolet-absorbing material running at the same rate as reference substrate material was cut out as a strip and eluted with methanol or methylene chloride:methanol (1:1) by descending chromatography. A second ultraviolet-absorbing area containing the more polar 20α-reduction product was located and a strip of the same size as that containing the original material was cut out and eluted as before. The eluated steroids were read in methanol against appropriate paper blanks in a Beckman DU spectrophotometer at a wavelength of 240 mμ. The relative concentrations of the eluates were calculated from the optical density readings based on the molar extinction value of cortisol ($\epsilon = 15{,}900$).

A 4% ethanolic phosphomolybdic acid reagent was used to detect ring A reduction. The spray revealed the presence of a still more polar compound (blue spot) which was not visible under the ultraviolet lamp.

The infrared spectra of many of the 20α- and 20β-epimers have been determined.

Experimental

Progesterone (obtained from the Schering Research Division) (50 mg) was dissolved in 3 ml ethanol and added to 125 ml of inoculated medium. Aliquots were removed after 1, 3, and 7 days. The products were separated by paper chromatography in benzene:hexane (1:2) – 80% methanol solvent system developed for 2 hours. The steroids were eluted from the paper strips with methylene chloride:methanol (1:1).

11-β-Hydroxyprogesterone (obtained from Elite Chemical Co.) (19.7 mg) was dissolved in 2 ml of ethanol and added to 45 ml of inoculated medium. Aliquots

*Obtained from the culture collection of the Northern Regional Research Laboratories (USDA) at Peoria, Ill., as *Rhodotorula longissima* NRRL No. Y2343.

were removed after 2, 7, 15, and 21 days. The products were separated in the heptane:benzene (1:1) – 70% methanol solvent system developed for 3½ hours. The steroids were eluted with methanol.

11α-Hydroxyprogesterone (obtained from Elite Chemical Co.) (24.4 mg) was added to 47.8 ml of inoculated medium with 1 ml ethanol. Aliquots were removed after 2, 7, and 14 days. The reaction products were separated in the toluene – 70% methanol system developed for 2½ hours.

17α-Hydroxyprogesterone (obtained from Schering Research Division) (50 mg) was added to 100 ml of inoculated medium. Aliquots were removed after 1, 2, and 5 days. Separation of products was achieved in the heptane: benzene (1:1) – 70% methanol solvent system in 2½ hours. Steroids were eluted from the paper with methylene chloride:methanol (1:1).

11-Ketoprogesterone (obtained from Elite Chemical Co.) (27.5 mg) was added in 1 ml ethanol to 47.8 ml of inoculated medium. Aliquots were removed after 2, 4, 7, and 14 days. The steroids were separated in the toluene – 70% methanol during a 2½-hour run. The steroids were eluted from the paper with methanol.

11-Keto-17α-hydroxyprogesterone (obtained from Sharp, Merck and Dohme Research Laboratories) (10 mg) was dissolved in 1 ml ethanol and added to 35 ml of inoculated medium. Samples were removed in 2 and 5 days and chromatographed in the toluene – 70% methanol solvent system for 2½ hours. Compounds were eluted with methanol.

11β,17α-Dihydroxyprogesterone (obtained from Sharp, Merck and Dohme Research Laboratories) (5 mg) in 0.5 ml ethanol was added to 17 ml of inoculated medium. Aliquots were taken after 2 and 5 days and chromatographed in the toluene – 70% methanol system for 2½ hours. Elution was carried out with methanol.

17β-Hydroxy-17α(iso)-progesterone (obtained from Elite Chemical Co. (26.4 mg) in 1 ml ethanol was added to 44.8 ml of inoculated medium. Samples were removed after 1, 2, 7, 14, and 21 days. Chromatography was carried out with the toluene – 70% methanol solvent mixture for 2½ hours.

Corticosterone (obtained from Mann Research Laboratories) (48 mg) was dissolved in 3 ml of ethanol and added to 100 ml of inoculated medium. Aliquots were taken after 13 and 32 days and chromatographed in toluene – 70% methanol for 2½ hours.

17α-Hydroxycorticosterone (hydrocortisone; obtained from Sigma Chemical Co.) (50 mg) was added to 100 ml of inoculated medium with 3 ml ethanol. Aliquots were removed after 13 and 32 days and chromatographed in the toluene – 70% methanol solvent system for 16 hours. Compounds were eluted with methanol.

11α-Hydrocortisone (obtained from Elite Chemical Co.) (19 mg) was added with 1 ml of ethanol to 47 ml of inoculated medium. Aliquots were taken at 2, 7, 15, 21, and 28 days and chromatographed in benzene:chloroform (1:1) – 70% methanol for 5 hours. Steroids were eluted from the paper with methanol.

11-Dehydro-17α-hydroxycorticosterone (cortisone; obtained from Mann Research Laboratories) (50 mg) was dissolved in 3 ml of ethanol and added to 100 ml of inoculated medium. Samples were taken at 13 and 32 days. Chromatography was carried out in toluene – 70% methanol for 5 hours. Steroids were eluted with methanol.

11-Desoxycorticosterone (obtained from Sigma Chemical Co.) (50 mg) was dissolved in 3 ml of ethanol and added to 90 ml of inoculated medium. Aliquots were taken after 1, 3, 7, 14, and 28 days. Chromatography was performed in the toluene – 70% methanol system for 3 hours.

11-Desoxy-17α-hydroxycorticosterone (Reichstein's substance S; obtained from Sigma Chemical Co.) (50 mg) was dissolved in 3 ml of ethanol which was added to 125 ml of inoculated medium. Aliquots were removed after 1, 3, and 7 days. The products were separated in the toluene – 70% methanol solvent system in 3½ hours. The steroids were eluted with methanol.

Results and Discussion

Sodium borohydride was employed to prepare several 20-dihydro-epimeric pairs of steroids (5, 6) which were then separated by paper chromatography (Table I). The microbiological reduction products of cortisone, Reichstein's

TABLE I

Paper chromatography of 20-dihydrosteroids

Compound	Solvent system	R_T (hours)*
11β,17α,20α,21-Tetrahydroxy-Δ⁴-pregnen-3-one (1)†	Toluene – 70% MeOH	.274
11β,17α,20β,21-Tetrahydroxy-Δ⁴-pregnen-3-one (2)	Toluene – 70% McOH	.342
17α,20α,21-Trihydroxy-Δ⁴-pregnen-3,11-dione (3)	Toluene – 70% MeOH	.930‡
17α,20β,21-Trihydroxy-Δ⁴-pregnen-3,11-dione (4)	Toluene – 70% MeOH	.779‡
11β,20α,21-Trihydroxy-Δ⁴-pregnen-3-one (5)	Toluene – 70% MeOH	.908
11β,20β,21-Trihydroxy-Δ⁴-pregnen-3-one (6)	Toluene – 70% MeOH	1.21
17β,20α,21-Trihydroxy-Δ⁴-pregnen-3-one (7)	Toluene – 70% MeOH	2.28
11β,20β,21-Trihydroxy-Δ⁴-pregnen-3-one (8)	Toluene – 70% MeOH	3.93
17α,20α-Dihydroxy-Δ⁴-pregnen-3-one (9)	Hexane – propylene glycol (30% MeOH)	.136
17α,20β-Dihydroxy-Δ⁴-pregnen-3-one (10)		.216
20α-Hydroxy-Δ⁴-pregnen-3-one (11)	Heptane – 80% MeOH	.512
20β-Hydroxy-Δ⁴-pregnen-3-one (12)	—	

*R_T = rate of migration in cm/hour. Intended primarily to show the relative mobility of epimeric pairs.
†(1, 2) 20-dihydrocortisols; (3, 4) 20-dihydrocortisones; (5, 6) 20-dihydrocorticosterones; (7, 8) 20-dihydro-11-desoxycortisols; (9, 10) 20-dihydro-17α-hydroxyprogesterones; (11, 12) 20-dihydro-progesterones.
‡Note that the α-epimer is the least polar. The α-dihydro-epimer was more polar than the β for all other epimeric pairs investigated.

substance S, and 17α-hydroxyprogesterone had the same chromatographic mobilities as the corresponding α-epimers prepared chemically. Since each of these 20α-dihydrosteroids was identified, they were not investigated further (6, 13). In order to extend the finding that the Δ⁴-3-ketonic steroid formed by the microorganism was always the 20α-dihydro-epimer, additional reduction products were identified. The microbiological reduction products of progesterone, 11-keto-17α-hydroxyprogesterone and 11β,17α-dihydroxyproges-

terone, were chromatographed in the heptane – 80% methanol, heptane: benzene (1:1) – 70% methanol, and toluene – 70% methanol solvent systems, respectively. The corresponding 20α- and 20β-dihydro-epimers, prepared by borohydride reduction, were chromatographed at the same time. The relative mobilities (α/β) of the respective epimeric pairs were .62, .81, and .75. The mobilities of the microbiological reduction products were identical with those of the α-epimers prepared chemically. After 2 hours at room temperature, the microbiologically produced 20α-dihydro-epimers of progesterone, 11-keto-17α-hydroxyprogesterone, and 11β,17α-dihydroxyprogesterone gave sulphuric acid chromogens with maxima at 292 and 400 mμ (very weak), 282 and 392 mμ, and 283 and 393 mμ, respectively. The sulphuric acid chromogens produced by the chemically prepared α-dihydro-epimers were identical with those obtained for the corresponding microbiological reduction products.

The characteristic of substrate specificity has been observed in steroid transformation reactions carried out by microbial enzymes (7). From the results contained in Tables II and III it is clear that the course of 20-keto reduction by *Rhodotorula longissima* is considerably altered by minor changes in the functional groups attached to the steroid molecule.

This may be elucidated by an examination of the data obtained for progesterone and its analogues.* After an incubation period of 7 days only 18% of the ultraviolet-light-absorbing material remained for progesterone. Of this amount, 30% was the 20α-hydroxy reduction product. For the same incubation period with 11β-hydroxyprogesterone, 31.8% of the original ultraviolet absorption was recovered of which there was 5.5% of the 20α-reduction product. On the other hand, incubation of 11-ketoprogesterone resulted in a recovery of 46.4% of the ultraviolet-absorbing material added to the incubation mixture. Of this amount 57.8% was the 20α-conversion product. In view of the difference in the amount of ultraviolet-light-absorbing material remaining, it would appear that 11-oxygenation was important in the preservation of the Δ^4-3-ketone structure. Moreover, the presence of a carbonyl group at C-11 appears to enhance 20-keto reduction, since 20α-reduced 11-ketoprogesterone was found in the reaction mixture as early as the second day. In contrast, although the hydroxyl group at C-11 seemed to increase ring A stability, a slower conversion rate to the 20α-reduction compound was obtained for 11β-hydroxyprogesterone. It follows then that addition of an 11β-hydroxyl group inhibits 20-keto reduction.

With the addition of a 17α-hydroxyl group to the progesterone molecule, a marked increase was noted in the rate of 20α-reduction. The conversion of 17α-hydroxyprogesterone to 17α,20α-dihydroxy-Δ^4-pregnen-3-one appeared to be complete in 24 hours. Further evidence of the hindering nature of a hydroxyl group at C-11 may be seen in the much slower reaction rate of

*For convenience in reference, those compounds which do not possess the hydroxyl at C-21 will be described as belonging to the "progesterone" series, and those compounds with the C-21-hydroxyl group as belonging to the "corticosterone" series.

TABLE II

Progesterone series

Ultraviolet-absorbing material remaining and 20α-reduction product formed after incubation with *Rhodotorula longissima* (%)

Incubation period (days)		P*	11β-OH-P	11α-OH-P	17α-OH-P	11-Keto-P†	11-Keto-17αOH-P	11β,17α-Di-OH-P	17β-OH-17α(iso)-P
1	U.V.	35.2			79.6				96.0
	20α	<5			94.2				None detected
2	U.V.		71.8	61.7	79.2	71.6	100	72.0	86.9
	20α		5.1	—	94.2	40.9	100	17.7	None detected
3	U.V.	24.3							
	20α	<5							
4	U.V.					67.6			
	20α					45.3			
5	U.V.				55.2		96.6	86.6	
	20α				96.8		100	46.0	
7	U.V.	18.4	31.8	10.5		46.4			72.8
	20α	30.0	5.5	+		57.8			None detected
14	U.V.			2.91		19.0			42.2
	20α			+		100			None detected
15	U.V.		9.6						
	20α		18.5						
21	U.V.		9.9						35.7
	20α		19.1						None detected

*P = progesterone.
†The Δ^4-3-ketonic reduction product was not proved to be 20α-hydroxy-11-ketoprogesterone.

TABLE III

Corticosterone series

Ultraviolet-absorbing material remaining and 20α-reduction product formed after incubation with *Rhodotorula longissima* (%)

Incubation period (days)		C*	17α-OH-C (cortisol)	11α-Hydro-cortisone	11-Dehydro-17α-OH-C (cortisone)	11-Desoxy-C	11-Desoxy-17α-OH-C (substance S)
1	U.V.					42.5	41.5
	20α					None detected	40.0
2	U.V.			77.8			
	20α			None detected			
3	U.V.					50.4	28.7
	20α					None detected	95.0
4	U.V.						
	20α						
5	U.V.						
	20α						
7	U.V.			73.3		46.1	17.4
	20α			None detected		None detected	100
13	U.V.	68.0	88.0		91.0		
	20α	None detected	None detected		50.6		
14	U.V.					16.9	
	20α					None detected	
15	U.V.			55.2			
	20α			None detected			
21	U.V.			48.9			
	20α			None detected			
28	U.V.			40.9		7.4	
	20α			13.8		None detected	
32	U.V.	13.3	66.7		72.4		
	20α	None detected	18.8		95.4		

*C = corticosterone.

11β,17α-dihydroxyprogesterone where only 46% of the remaining steroid was 11β,17α,20α-trihydroxy-Δ^4-pregnen-3-one after a reaction period of 5 days. On the other hand, 100% conversion of 11-keto-17α-hydroxyprogesterone was effected in 2 days and the product was recovered in nearly quantitative yield even after 5 days' incubation.

The corresponding compounds of the corticosterone series (i.e. C-21-hydroxysteroids) were also subjected to reduction by this method in order to determine if the results obtained with compounds of the progesterone series had more general application. In agreement with the expectations based on the findings with progesterone and its analogues a good recovery of ultraviolet-absorbing material was found with cortisone and hydrocortisone even on prolonged incubation (32 days). Again, the 11-keto compound, cortisone, was more readily converted to the 20α-dihydro compound than was its 11β-hydroxy counterpart, hydrocortisone. It is worthy to note that a change in the hydroxyl group at C-11 from the normal β in hydrocortisone to an α-configuration decreased ring A stability on prolonged incubation. This phenomenon was observed with 11α-hydroxyprogesterone as well.

As a rule, the steroids of the C-21-hydroxy series possessed a greater degree of ring A stability than did the 21-desoxy analogues, but reduction of the C-20 carbonyl proceeded at a slower rate. For example, the difference in structure of 11-keto-17α-hydroxyprogesterone and cortisone resides in the absence of a hydroxyl group at the C-21 position in the former. However, the rate of formation of 20α-conversion product is many times slower for cortisone. It is probable, then, that hydroxylation at C-21 may contribute to ring A stability, but at the same time hinder reduction of the 20-ketone through an alteration of the spatial relationship in the steroid molecule in such a way that the points of reactivity between enzyme and substrate may be blocked. This may be further exemplified in the contrast in reactivity of progesterone and 11-desoxycorticosterone. Both compounds lack the 11-oxygen function and hence are susceptible to ring A instability. However, 11-desoxycorticosterone is at least twice as stable as progesterone to the loss of the Δ^4-3-ketone entity, the difference presumably being due to hydroxylation at C-21. On the other hand, in the absence of the accelerating 17α-hydroxyl group, and by reason of the presence of the strongly inhibiting 21-hydroxyl group, no evidence of 20α-reduction could be detected even on prolonged incubation of 11-desoxycorticosterone. Such also was the case with corticosterone. The fact that 20-keto reduction was achieved with hydrocortisone, cortisone, and Reichstein's substance S was no doubt due to the influence of the strongly activating 17α-hydroxyl group. That the spatial relationship of the various groups on the steroid molecule is the determining factor in these enzymic reactions is verified by the inability of the yeast to reduce 17β-hydroxy-17α(iso)-progesterone even on prolonged incubation.

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PILOCARPINE-INDUCED CONVULSIONS AND DELAYED PSYCHOTIC-LIKE REACTION[1]

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Abstract

The subcutaneous injection into albino rats of pilocarpine hydrochloride at doses in the range of the median lethal dose produced two types of acute toxicity syndromes, an immediate muscarinic convulsive reaction and a delayed psychotic-like reaction. The $LD_{50} \pm S.E.$ of the immediate reaction was estimated at 642 ± 90 mg per kg and that of the delayed reaction at 430 ± 126 mg per kg. The mean $\pm$ standard deviation time to death in the immediate reaction was 34 ± 20 minutes and in the delayed reaction 4.4 ± 2.5 days. The cause of death in the immediate reaction was postconvulsive respiratory paralysis following marked muscarinic activity and accompanied by hypothermia and by vascular congestion of the brain, heart, lungs, and kidneys. The muscarinic and convulsive signs disappeared in survivors by the second day and were replaced by either a depressant or excitant type of neuropsychosis characterized by disorientation and cataleptiform trances. Animals with the depressant type tended to die in hypothermic stupor with capillary vasodilatation and(or) hemorrhage and(or) other toxemic changes in many organs. Animals with the excitant type of reaction tended to survive with degrees of psychotic-like behavior persisting for a month.

Introduction

During the course of studies, in this department, on the role of the autonomic nervous system in the etiology of fibrocystic disease of the pancreas (1), it became necessary to know the effects, in animals, of single lethal and near-lethal doses of pilocarpine. A review of the literature revealed that there was limited information available upon this subject, an observation which is not unusual since pilocarpine was introduced in the latter part of the last century when extensive toxicity studies of new drugs were not required. Consequently a systematic study of the effects of subcutaneous injections at the range of the median lethal dose was undertaken in the albino rat. The clinical and pathological syndrome so produced differed considerably from that which had been anticipated.

Sollmann (2) describes death from pilocarpine as due to paralysis of the heart or edema of the lung following signs of marked parasympathomimetic activity. A similar statement occurs in a textbook published 30 years earlier (3). Evidence reviewed by Spector (4) indicates that "lethal doses" of pilocarpine have been found between 120 and 353 mg per kg body weight given intravenously to rabbits and pigeons. Rabinovitch *et al.* (5) found that doses of the order of 40 to 50 mg per kg given intravenously killed some 10 to 20% of mice. Hollander and Stein (6) recorded signs of stimulation of the central nervous system in dogs given pilocarpine hydrochloride subcutaneously in doses of

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0.5 and 1.0 mg per kg. Of these signs, Hollander (7) found that vomiting in dogs had been reported in 1875 at the time jaborandi was first introduced in France. According to Borison (8), the emetic action of pilocarpine in cats is due to stimulation of the basal cortex of the frontal lobe, possibly aided by an action on the emetic chemoreceptor trigger zone in the area postrema of the medulla oblongata. Sollmann (2) states that the effects of pilocarpine on the central nervous system are mainly depressing but that tremors and feeble convulsions can be produced.

Statements of earlier observers have varied, therefore, on the effects, other than parasympathomimetic, of large doses of pilocarpine. The differences appear to have been related to the ability (cats and dogs), or inability (mice and rats), of the experimental animal to vomit under the influence of the drug, and to the period of observation. Most investigators appear to have confined their period of observation to a few minutes or a few hours after administration of pilocarpine. In such an experimental design, the delayed reaction noted below would not be recorded.

Methods

The experimental animals were male albino rats of a Wistar strain which have been bred in the animal quarters of the Department of Pharmacology at Queen's University since 1937. They were young animals of 250 to 400 g body weight and were fed Purina fox chow checkers and water ad libitum. Each rat was housed singly in a metabolism cage for observation of the effects of pilocarpine. Pilocarpine was used in the form of the hydrochloride, which is more soluble in water than the nitrate, as it was anticipated that very large doses might be required. Pilocarpine hydrochloride was kept in a desiccator since it is hygroscopic. Sterile solutions in distilled water were prepared just before use and injected subcutaneously in a volume of 5 ml per kg body weight. Doses of 200, 400, 550, 600, 650, 700, 800, and 1000 mg per kg were each given to 12 to 14 animals except in the cases of the highest and lowest doses whose numbers were restricted to two per dosage group. Observations and measurements noted below were made daily for periods up to 10 days and then casually for a further month, after the technique of Boyd (9).

Results

There were 53 deaths amongst the 80 animals given pilocarpine. As illustrated in Fig. 1, the intervals to death fell into two separate frequency distributions, with 39 deaths at 6 to 89 minutes and 14 at ½ to 8 days. The former will be referred to as immediate deaths and the latter as delayed deaths. The mean $\pm$ standard deviation time to immediate death was 34 ± 20 minutes and to delayed death 4.4 ± 2.5 days.

Dosage plotted against percentage of mortality, with not more than one entry at 0% or 100% mortalities, yielded linear regressions as shown in Fig. 2. The estimating equation, $\pm$ its standard error, for immediate death, calculated by

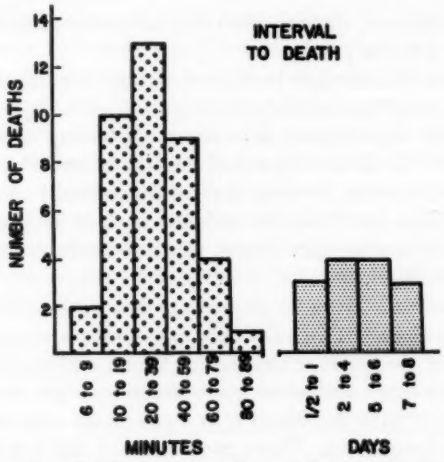

FIG. 1. The frequency distribution of deaths at intervals after administration of pilocarpine. Note variations in the time scale of the abscissa.

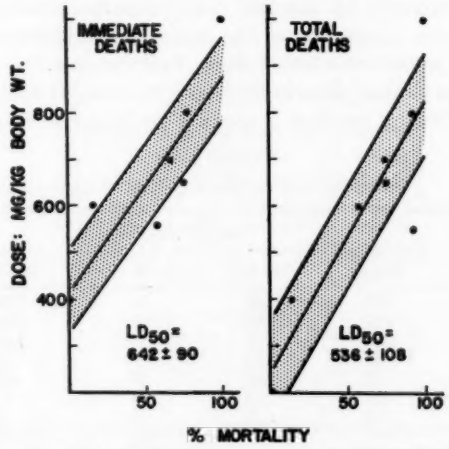

FIG. 2. The regression of dosage on percentage of mortality for immediate (6 to 89 minutes) and total deaths.

the method of least squares (10), was $Ye = (411+4.62X) \pm 90$ and, for total deaths, $Ye = (249 \pm 5.73X) \pm 108$. From these equations, the median lethal dose $\pm$ standard error for immediate death was estimated at 642 ± 90 and for total deaths 536 ± 108 mg per kg body weight given subcutaneously. It was not possible to calculate the LD_{50} for delayed deaths by the same method since all except two of these occurred in survivors to immediate death. Assuming that the LD_{50} for total deaths was the arithmetic mean of the LD_{50} for immediate,

and the LD_{50} for delayed, deaths, then the LD_{50} for delayed deaths may be estimated at 430 ± 126 mg per kg.

A summary of the clinical signs in animals dying by immediate death is given in Table I. Within ½ to 3 minutes of drug injection, all animals began to salivate profusely and had a loose bowel movement containing mucus. At the same time, animals given the lesser doses had chromodacryorrhea and nystagmus. There next occurred a series of clonic convulsions usually at intervals of a few minutes. During those intervals, the animals became progressively more listless, apathetic, and hypothermic. Death was due to respiratory failure following a terminal convulsion.

At autopsy on animals dying by immediate death, the renal veins appeared grossly engorged with blood. Microscopic examination revealed capillary and venous congestion in the renal cortex, lungs, heart, and meninges. There were small thrombi in the lungs and some hemorrhage into the alveoli. The splenic sinuses were engorged with red blood cells and splenic venous blood contained a marked excess of leucocytes. There were areas of vacuole-like formation in the hepatic cells and in the zona fasciculata of the adrenal cortex.

The cause of immediate death, therefore, was respiratory failure following convulsions, accompanied by marked parasympathomimetic activity, hypothermia, and vascular congestion of the brain, heart, kidneys, and lungs, due to subcutaneous injection of a lethal dose of pilocaprine.

A summary of the clinical signs in animals with delayed deaths is presented in Tables I and II. During the first 2 hours after injection of pilocarpine, their

TABLE I

The percentage incidence of clinical signs

Clinical sign	Immediate deaths	Delayed deaths		Survivors	
		1st day	2nd–7th days	1st day	2nd–7th days
Sialorrhea	100	100	0	100	0
Mucous diarrhea	100	100	0	100	0
Clonic convulsions	100	85	0	100	0
Listlessness	69	54	75	33	0
Chromodacryorrhea	18	46	0	67	0
Impaired righting reflex	18	69	0	12	0
Nystagmus	10	0	0	0	0
Ataxia	—	—	25	—	0
Upright stance	0	0	25	4	14
Muscular twitching	—	—	0	—	14
Anal discharge	—	—	50	—	29
Spacial disorientation	—	—	25	—	43
Normal	0	0	0	0	43
Irritability	—	—	0	—	57

response was similar to that of animals which died at this time. They survived immediate death and remained apathetic and stuporous during the remainder of the first day.

From the second day and until death, signs of convulsant and parasympathomimetic activity had disappeared. The animals were listless and often

ataxic. Some of them went into what was termed a 'praying kangaroo trance.' These rats sat upright with their heads bowed in a withdrawn trance from which they could not readily be aroused. Others appeared to have a special disorientation; they would walk to the edge of the laboratory table and fall off. There was no evidence of blindness since if thoroughly frightened they would run away, avoiding obstacles in their flight. Histologically the retina appeared normal. Their appetite was decreased, their food consumption was about half that of the controls, and their weight was reduced (Table II). They had oligodipsia, increasing oliguria, and increasing hypothermia (Table II). In those rats seen at the time of death, death was due to respiratory failure in a deep stupor.

TABLE II

Shifts in body weight, food and water intake, urine volume, and colonic temperature
(The results are expressed as mean ± standard deviation)

Group	N	Days after pilocarpine		
		1	3	5
	Body wt. (g increase (+) or decrease (−))			
Nonsurvivors	53	−35.2±13.0	−51.8±29.6	−65.4±36.0
Survivors	27	−24.5±18.2	−25.1±33.6	−17.8±41.2
Controls	31	+ 2.0± 4.6	+ 4.9± 5.3	+ 8.0± 6.8
	Food intake (g chow/kg body wt./24 hours)			
Nonsurvivors	53	17.4±33.2	36.2±58.7	32.4±40.6
Survivors	27	65.2±58.8	94.1±79.4	123.3±81.1
Controls	31	80.3±11.4	70.8± 8.5	71.0± 7.7
	Water intake (ml/kg body wt./24 hours)			
Nonsurvivors	53	48.2±63.6	56.2±89.1	45.8±51.0
Survivors	27	111.5±97.1	156.0±83.9	214.3±83.0
Controls	31	104.8±18.2	89.0±13.8	88.7±19.6
	Urine volume (ml/kg body wt./24 hours)			
Nonsurvivors	53	8.8±4.4	9.6±11.8	3.8± 5.2
Survivors	27	12.5±8.3	7.6± 5.0	31.5±44.8
Controls	31	23.3±6.9	28.9± 7.2	35.1± 9.0
	Colonic temperature (°F)			
Nonsurvivors	53	99.4±1.0	96.4±1.9	94.1±6.0
Survivors	27	99.2±1.6	97.9±2.5	100.1±0.7
Controls	31	99.3±0.7	99.2±0.7	99.2±0.5

Gross pathological examination after delayed death revealed hemorrhagic areas in the stomach and small bowel, caseous areas in the liver, enlarged and kidney-colored adrenal glands, and a small spleen and thymus gland.

Microscopic examination at autopsy, after delayed deaths, revealed considerable pathological changes in the gastrointestinal tract. There were areas of venous congestion in the submucosa of the cardiac stomach. In the pyloric stomach capillary dilatation occurred in the lamina propria with capillary hemorrhages oozing into the mouths of the gastric glands. A similar capillary congestion was present in the lamina propria of the intestinal villi, especially in the ileum. There were hemorrhages into the submucosa of the cecum. The vascular lesions were considerably less marked in the large bowel.

The caseous areas seen on gross examination of the liver proved to be areas of complete necrosis of hepatic cells with leucocytic infiltration. Otherwise the sinusoids and central lobular veins of the liver were engorged with erythrocytes. The small spleen was due to contracture of the red pulp, leaving the white pulp prominent. The sinusoids of the zona glomerulosa of the adrenal cortex were distended with packed red blood cells. The atrophy of the thymus gland seen on gross examination was due to loss of thymocytes; this loss left the reticular cells and Hassall's bodies prominent and having what appeared to be a residual congestion of capillaries and veins.

The capillaries of the renal cortex were distended with erythrocytes and there was some granular change in the cells of the proximal and distal convoluted tubules. There were congestion with edema, small hemorrhages into the alveolar sacs, and occasional arteriolar thrombi in the lungs. Cardiac muscle was congested and the A disks of the cross striations were granular. Capillary vasodilatation was also present in the meninges and in areas throughout the cerebrum. The cerebellum and medulla oblongata appeared normal. In the testicles, there were areas of degeneration of the spermatogonium, the primary and secondary spermatocytes, and sperm. The Sertoli cells appeared normal. Some tubules of the epididymus contained what appeared to be degenerated spermatocytes scattered amongst the sperm.

The cause of delayed deaths, therefore, was respiratory failure, consequent to increasing encephalopathy, accompanied by congestion, with and without capillary hemorrhage, in the gastrointestinal tract, liver, adrenal cortex, thymus, renal cortex, lungs, heart, and brain, and degenerative changes in various organs, due to subcutaneous administration of a lethal dose of pilocarpine.

A summary of clinical signs in survivors is given in Tables I and II. Their reaction during the first few hours after drug administration was similar to that of the nonsurvivors except that they were less depressed after clonic convulsions and the incidence of chromodacyorrhea was higher. On the second and subsequent days until recovery, survivors became excited rather than depressed. Some degree of excitement was present for as long as 6 weeks after administration of pilocarpine. Some of the survivors had signs of psychotic behavior in the form of special disorientation and the assumption from time to time of the 'praying kangaroo trance.'

By the end of 1 week after drug administration, many survivors had developed voracious appetites and were rapidly gaining weight. At the same time they drank large volumes of water and had a slight rise in colonic temperatures (Table II). At autopsy 4 to 6 weeks after receiving pilocarpine, the organs appeared grossly normal except that the spleen was small.

Discussion

The results of these experiments demonstrated that pilocarpine given subcutaneously in doses within the range of the median lethal dose produced two

types of toxicity syndromes in albino rats.

One syndrome was called the immediate reaction since death occurred within minutes of the time of injection. The $LD_{50} \pm$ S.E. was estimated to be 642 ± 90 mg per kg body weight. These doses of pilocarpine hydrochloride produced marked parasympathomimetic stimulation followed by clonic convulsions, capillary and venous congestion of the brain, heart, lungs, and kidneys, and postconvulsive respiratory failure. As noted in the Introduction, certain aspects of this immediate reaction have been previously described (2, 3). Fellows and Livingston (11) reported an identical type of clinical reaction in rats following subcutaneous injection of lethal doses of the parasympathomimetic drug furtrethonium or Furmethide. Intravenous administration of lethal doses of anticholinesterases such as sarin and tabun produces a somewhat similar clinical syndrome with death due to asphyxia or cardiovascular collapse following convulsions (12, 13).

The second syndrome was called the delayed reaction since it appeared several days after subcutaneous administration of pilocarpine hydrochloride. The $LD_{50} \pm$ S.E. was estimated at 430 ± 126 mg per kg body weight. The animals survived the primary reaction and developed a neuropsychosis characterized by listlessness, disorientation, and cataleptiform trances. Capillary congestion and hemorrhage occurred in many organs of the body. The animals died in hypothermic stupor at periods up to 8 days after drug administration. Survivors had a similar neuropsychosis but tended to be excited and apprehensive rather than listless and depressed.

The psychotic-like reaction did not appear to be a sequela of the convulsions since similar convulsions produced by correspondingly large doses of other drugs, such as acetylsalicylic acid (14), caffeine (15), and benzylpenicillin (16), are not necessarily followed by a similar neuropsychosis. A delayed psychotic-like reaction has not been described as part of the reaction to lethal and near-lethal doses of most older muscarinic parasympathomimetic agents, possibly because the syndrome was not followed in animals for a sufficiently long period of time and because these older drugs are used in limited amounts in man. Hart (17) has noted, however, that recovery from anticholinesterase nerve-gas poisoning may be accompanied by anxiety, tension, emotional instability, and irrational behavior lasting a few days.

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ISOLATION AND COMPOSITION OF AVIAN EGG YOLK GRANULES AND THEIR CONSTITUENT α- AND β-LIPOVITELLINS[1]

R. W. BURLEY[2] AND W. H. COOK

Abstract

The yolk granules from hen's egg represent on a dry basis 23% of the yolk solids, and they contain about 90% of the protein phosphorus, 95% of the iron, and nearly 70% of the calcium in yolk. Ultracentrifugal and other analyses on solutions of the granules show that they are 70% α- and β-lipovitellin in an approximate ratio of 1:1.8, 16% phosvitin, and 12% low-density lipoprotein. The properties and composition of the two lipovitellins isolated from the granules are the same as those isolated from solutions of whole yolk. Further purification reduces the protein phosphorus in α-lipovitellin to 0.50% and in β-lipovitellin to 0.27%, and this confirms that α-vitellin has a higher phosphorus content. Experiments at low temperature suggest that phosvitin exists in the granules as a high molecular weight complex.

Introduction

Avian egg yolk contains a variety of microscopic particles belonging to two main groups: the "yolk globules", which are large particles, many of them resembling oil droplets; and the "granules", which are much smaller and more uniform in size but less regular in shape (1). Few critical studies have been made on the isolation and composition of the granules. They are known to sediment on centrifugation, and lipid, phosphorus, and other analyses on the fractions suggest that the sedimenting material contains lipoprotein and phosphoprotein (2). Recent ultracentrifugal studies on solutions of the granules (3) leave doubt about which of the seven proteins and lipoproteins that have been isolated from egg yolk (4, 5, 6, 7) are present in the granules, although it may be inferred that they contain the lipovitellins, phosvitin, and some of the low-density lipoprotein fraction (LDF).

More detailed investigations on the composition of the granules and on the isolation and characterization of the granule proteins were undertaken primarily to establish the source of the two newly discovered components, α- and β-lipovitellin (6, 7), and their relation to the other protein components found in solutions of whole yolk. As additional information was required to establish the protein phosphorus and nitrogen contents of the two lipovitellins, an improved procedure was developed for their purification. This is described and analytical results are reported.

Methods

Phosphorus was estimated by the method of King (8), which extensive tests in this laboratory have shown to give the same results as gravimetric and more

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elaborate colorimetric methods. Nitrogen was estimated by the micro-Kjeldahl procedure, and sulphur by the method of Schöniger (9). Calcium and iron were estimated spectrographically. Protein and lipoprotein concentrations were determined by exhaustively dialyzing a portion of the solution against water and drying the solid to constant weight at 105° C. Drying over phosphorus pentoxide *in vacuo* gave the same results.

Total lipid was estimated by the method of Bligh and Dyer (10) as follows: To a known amount of lipoprotein (0.2 to 0.4 g) in 10.0 ml of *M* sodium chloride or 0.7 *M* sodium acetate, 12.5 ml of chloroform and 25 ml of methanol were added. After the mixture had been allowed to stand overnight at room temperature 12.5 ml of chloroform and an equal amount of water were added. The precipitated protein was filtered off, washed once with chloroform and then thoroughly with water, and weighed after being dried either at 105° C or over phosphorus pentoxide. The amount of lipid was measured by noting the volume of the chloroform phase and drying a portion of it to constant weight. This method has the advantage that the mixture is homogeneous initially.

The pH 9.0 sodium barbiturate buffer (6) contained 0.25 *M* sodium chloride and had an ionic strength of 0.3. The pH 6.8 phosphate buffers used for chromatography were prepared by diluting a 2 *M* solution containing 136.1 g KH_2PO_4 and 228.2 g $K_2HPO_4.3H_2O$ per liter.

Preparative separations were made with a Spinco Model L centrifuge using rotor No. 30. Sedimentation studies were made in a Spinco Model E ultracentrifuge by procedures already described (6) unless otherwise stated. Normally, double-sector cells, a speed of 50,740 r.p.m., and a temperature of 20° C were used. For the measurement of protein concentrations, ultracentrifuge patterns were enlarged 14 times linearly and the areas of the peaks measured with a mechanical integrator. Areas were converted to concentrations from similar measurements on solutions containing known amounts of each protein.

Partial specific volumes were measured at 20.0° C by means of a MacInnes magnetic float apparatus (11, 12) in *M* sodium chloride.

Experimental and Results

Isolation of Granules

Most of the procedures used previously for the initial concentration of the granules involved centrifuging whole yolk or yolk diluted with isotonic salt solution (2, 3, 13), and several modifications of these procedures were tested in preliminary experiments. The granules were found to sediment rapidly when whole yolk was centrifuged at 0° C, but prolonged centrifugation was necessary to pack them sufficiently to permit sharp separation. Granules recovered after 16-hour centrifugation at 30,000 r.p.m. at 0° C were badly contaminated with yolk globules, although better separation was achieved at higher temperatures. When yolk was diluted with isotonic saline or water, only 10-minute centrifuging at 29,000 r.p.m. was required to sediment and pack the granules: this

procedure was preferred to prolonged centrifugation of whole yolk. Apart from a tendency to cause groups of granules to cluster, dilution had no apparent effect and granules having similar properties when dissolved were obtained with either diluent. Microscopic examination of the sedimented pellet showed that it contained very few globules. Recentrifuging the supernatant for an additional 20 minutes sedimented a loose deposit containing particles resembling globules, or remnants of globules, but a negligible amount of material resembling granules. Evidently the granules can be separated from diluted yolk, although some globules also sediment. It was therefore necessary to wash the granules and this was done by grinding the adherent pellet in the medium used previously as diluent and recentrifuging to remove the remaining globules and soluble contaminants such as the livetins.

Grinding the isolated granules in water caused only a 2% loss of weight and this probably represented contamination by other yolk constituents. About half the dissolved material was not dialyzable and ultracentrifugation indicated that it was chiefly LDF. The dialyzable material was acidic and appeared to have the property of dispersing aggregated granules. It was not examined further.

The following procedure was used for the isolation of the yolk granules. Most operations were performed at room temperature, except preparative centrifugation, which was always done at 0° C, and some low-temperature experiments described later. Yolk (60 ml) from fresh hen's eggs was diluted with an equal volume of 0.16 *M* sodium chloride, or with water in a few experiments, and centrifuged for 10 minutes at 29,000 r.p.m. The supernatant liquid was decanted and the pellet of granules thoroughly dispersed in 120 ml of 0.16 *M* sodium chloride or water by grinding the mixture in a mortar. The granules were then recovered by centrifuging as before. Solutions of the granules were usually prepared at room temperature and stored at 0° C.

Properties and Chemical Composition of Granules

The granules were insoluble in water and subsequent analyses (Table I) showed that they lost a negligible proportion of their calcium, iron, or phosphorus after they had been washed several times in water. They could be dissolved completely in sodium chloride solutions of 0.3 *M* or higher but prolonged stirring was necessary below 1 *M*. Several other neutral salts tested had a similar solvent power.

Granules sedimented from undiluted yolk contained 44% of water and those isolated after dilution had 48–52% of water at all stages of preparation. The dry weight of granules isolated from 60 ml of yolk was 6.8 g. In agreement with previous results (2), this is about 11.5% by weight of the original yolk and about 23% by weight of the yolk solids.

Representative results of lipid, calcium, iron, and phosphorus analyses made on the granules and on whole yolk are given in Table I, together with comparable quantities reported by others. Comparison of the lipid contents of

TABLE I

Lipid and elementary analysis of granules and whole yolk solids (dry weight basis)

Sample	Total lipid (%)	Ca (%)	Fe (%)	Total phosphorus (%)	Protein phosphorus (%)
Granules from:					
(*a*) Undiluted yolk, not washed	45	0.86*	0.24*	—	—
(*b*) Yolk diluted with water, not washed	31	—	—	—	1.98
(*c*) As (*b*), one wash with water	30	0.86	0.29	2.10	2.02
(*d*) As (*b*), three washes with water	29	0.88	0.29	—	2.04
(*e*) Yolk diluted with 0.16 *M* NaCl, not washed	—	—	—	—	2.04
(*f*) As (*e*), one wash with 0.16 *M* NaCl	30	0.80	0.29	2.10	1.98
(*g*) As (*e*), three washes with 0.16 *M* NaCl	—	—	—	—	2.04
(*h*) As (*f*), but different batch of yolk	30	—	—	—	1.93
Reported by others					
Schmidt *et al.* (2)	28	—	—	—	2.05
Schjeide and Urist (3)	—	(0.50±0.1)†	—	—	—
Whole yolk (values in parentheses from others (4))	(66)	0.29	0.007	(1.18)	(1.09)

*Corrected to 30% lipid content for comparison.
†On wet basis, moisture content not reported.

granules isolated from diluted and undiluted yolk shows that the separation of the granules and the LDF was facilitated by dilution. It was found that results for different preparations sometimes varied beyond the analytical errors, owing to variations in the procedures or possibly to a difference between eggs. For this reason, granules prepared from a common pool of yolk were used to assess the effect of the isolation and washing procedures (samples (*a*) to (*g*) in Table I). Washing with 0.16 *M* sodium chloride reduced the calcium content slightly, presumably by ion exchange.

Although the analyses of the granules given in Table I were undertaken to determine the effect of different isolation procedures, they can usefully be compared with analyses of whole yolk solids. Evidently the granules, which, as already mentioned, contain 23% of the yolk solids, contain 10% of the yolk lipids, 68% of the calcium, 95% of the iron, 41% of the total phosphorus, and 91% of the protein phosphorus. The estimate of the proportion of protein phosphorus is less reliable since it depends on the proportions of protein in whole yolk (taken as 33%) and in the granules (70%). Nevertheless our estimates agree with those of others (4, 13) in suggesting that the granules contain 90 to 97% of the protein phosphorus in yolk. The supernatant fluid from which the granules had been removed contained 0.15% of protein phosphorus on a dry basis, which corresponds to 7% of the total protein phosphorus and confirms the above estimates.

Preparation and Composition of the Granule Proteins

Granules from 50 ml of yolk were dissolved in 50 ml of 1 *M* sodium acetate by grinding in a mortar. The cloudy solution was centrifuged at 0° C for 16 hours at 30,000 r.p.m. and the floating material (LDF) removed after cutting off the top of the plastic tubes with a Spinco tube slicer. After the yellow supernatant solution had been adjusted to 0.7 *M* sodium acetate and 100 ml volume, it was applied to a column (2.5×50 cm) of Dowex 1 (X2, 200–400 mesh) that had been equilibrated at 4° C with 0.7 *M* sodium acetate and was eluted with the same solvent at a rate of 1 ml/minute or less. The lipovitellins were not retained under these conditions and emerged with the front in about the same volume as they were applied.

The solution of the mixed lipovitellins from the resin column was dialyzed against 0.2 *M* potassium phosphate buffer (pH 6.8) to displace most of the acetate and then applied to a column (4×20 cm) of hydroxyapatite (7). After being washed with at least 100 ml of the same buffer, the β- and α-lipovitellins were eluted with 0.6 *M* and 2 *M* phosphate buffer, respectively, dialyzed against 0.7 *M* sodium acetate, and passed down another Dowex 1 column (as above) to remove traces of phosphate and phosvitin.

This isolation procedure reduced the protein phosphorus contents of the lipovitellins to low values (Table II) which remained constant after further chromatography on Dowex 1 or hydroxyapatite columns, but the yields were

TABLE II

Analyses on lipovitellins

	α-Lipovitellin		β-Lipovitellin	
	This work (%)	Bernardi *et al.* (7) (%)	This work (%)	Bernardi *et al.* (7) (%)
Total lipid	22.2	20	21.7	20
Phospholipid*	11.6	11.0	11.6	(12.4)
Total phosphorus	0.91	1.40	0.65	0.95
Phosphorus in lipid	2.1	2.2	2.1	2.7
Phosphorus in protein	0.50	1.20	0.27	0.45
Nitrogen in protein	16.2	17.2	16.3	17.2
Sulphur in protein	1.14	1.44	1.21	1.20

*Lipid phosphorus × 25 except value in parentheses obtained by chromatography.

only about 60% of expectation. All steps appeared to contribute to the total loss and there was no evidence that unidentified constituents were removed during treatment, although this is possible.

Phosvitin was eluted from the first Dowex 1 column with 3 *M* sodium acetate. The eluate was collected in 15-ml fractions and the phosvitin located by absorption measurements at 280 mμ. After sodium acetate had been removed by dialysis, the solution was passed down a column (2.5×20 cm) of Amberlite 120 (X8, 200–400 mesh) in the hydrogen form. Phosvitin was not retained on this column. It was eluted with water and the solution freeze-dried. Protein and lipoprotein contaminants were retained as a whitish band at the top of the

column. Yields of phosvitin were only about 10% of expectation but elution of the first Dowex 1 column with 1 *M* magnesium acetate (14) increased the yield to about 40%. The reasons for the low yields are still under investigation, since this protein may be heterogeneous. All results reported here were from the first phosvitin fraction, i.e. eluted with 3 *M* sodium acetate.

Lipid and elementary analyses on the lipovitellins from one batch of granules are given in Table II together with the corresponding values already reported (7). Other batches of granules gave results in good, but seldom exact, agreement. Phosphorus values for β-lipovitellin, for example, ranged from 0.24% to 0.30%. Both lipovitellins contained slightly more lipid and less protein nitrogen than reported previously, but the most remarkable difference is the lower protein phosphorus in the new preparations. The earlier finding that α-vitellin contains more phosphorus than β-vitellin (7) has, however, been confirmed and the new values, in conjunction with the proportions of α- and β-lipovitellin given in Table V, are consistent with the amount of protein phosphorus found in unfractionated lipovitellin (0.37%).

It is possible that contamination by phosvitin was responsible for the higher values for phosphorus reported previously in α- and β-lipovitellin; alternatively, lipovitellin phosphorus might have been removed by the anion-exchange resin used in the new procedure. In agreement with the first alternative, a much lower serine concentration has been found in the new preparations. Serine and other amino-acid analyses will be reported in a later communication.

The phosvitin isolated from the granules contained 12.9% of nitrogen and 8.5% of phosphorus, uncorrected for ash (6.5%). Analytical results for phosvitin reported in the literature are extremely variable, especially in the amount of phosphorus for which values of from 7.5 to 10.4% have been found (15–18). Most previous preparations had about the same nitrogen as reported here but a higher phosphorus content. The presence of contaminants may account for the variability, although it is possible that phosvitin is heterogeneous and contains fractions with differing phosphorus content.

The other major constituent of the granules, the LDF, was not examined in detail. It contained at least 88% of lipid, and closely resembled the LDF from the rest of the yolk in appearance, although it was more difficult to disperse in salt solutions.

Identification of Granule Proteins

The ultracentrifugal patterns of a solution of yolk granules in pH 9.0 buffer at 20° C are shown in Fig. 1A. In this solvent four boundaries are evident. The identification of the three upper boundaries as phosvitin (PHOS), dissociated β-lipovitellin (β/2), and a mixture of α- and β-lipovitellin in the associated form (α + β) depends on the sedimentation coefficients and other properties of the isolated proteins given later. The inverted boundary represents the LDF because it was absent when this was removed and its removal did not affect the pattern of the remaining proteins (Figs. 2G and 2H). Figure 1B shows patterns

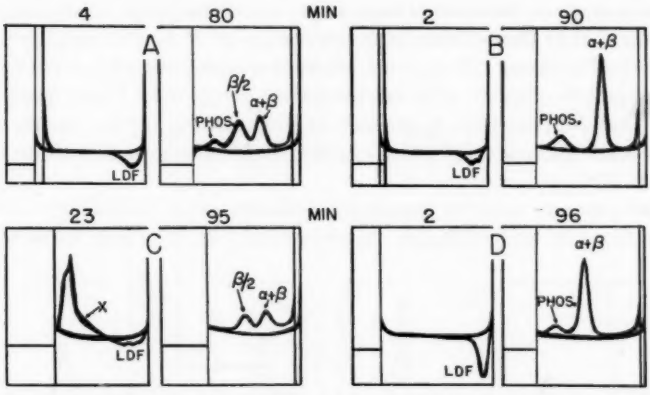

FIG. 1. Ultracentrifuge patterns of egg yolk granules dissolved in pH 9.0 buffer (A and C), and in *M* NaCl (B and D). A and B measured at 20° C, and C and D at 4° C. Sedimentation from left to right.

for granules dissolved in 1 *M* sodium chloride (pH 6.4). Under these conditions β-lipovitellin does not dissociate and, as expected from the above identification, boundary $\beta/2$ is absent.

As already described, several of the preparative steps were performed at 0° C, but the isolated granules were dissolved at room temperature and the analytical sedimentations were performed at 20° C. In order to determine the effect of temperature, similar measurements were also made on solutions of granules prepared as rapidly as possible at 0° C and sedimented at 4° C. In a typical experiment, 50 minutes was required for the isolation of the granules and 40 minutes from the addition of solvent until the rotor had reached full speed. During this period the temperature of the granules and their solutions did not exceed 4° C.

Typical ultracentrifugal patterns obtained in this way with samples examined at 4° C in pH 9.0 buffer and 1 *M* sodium chloride are shown in Figs. 1C and 1D. At pH 9.0 the phosvitin boundary was absent and a fast ill-defined boundary (X) appeared (Fig. 1C) which soon subsided and did not form a discrete peak. In 1 *M* sodium chloride the pattern at 4° C (Fig. 1D) was the same as that at 20° C (Fig. 1B). Evidently phosvitin occurs in the granules as part of a complex that disintegrates slowly, or not at all, at 4° C in pH 9.0 buffer but very rapidly in *M* sodium chloride at this temperature.

In the ultracentrifugal patterns at low temperature the lipovitellin peaks are the same as at 20° C, i.e. the dissociated β-lipovitellin peak, $\beta/2$, is present at pH 9.0 (Fig. 1C) but not at pH 6.4 (Fig. 1D). In order to determine whether this behavior was observed because β-lipovitellin was present in the granules in an *associated* form that dissociated rapidly on dissolution at pH 9.0, or in a *dissociated* form that associated rapidly in *M* sodium chloride (pH 6.4), measure-

ments were made on the rates of association and dissociation at low temperature. It was found that dissociation of β-lipovitellin at 4° C was rapid, equilibrium being reached in about 120 minutes, whereas samples initially at pH 9.0 showed little change 120 minutes after neutralization to pH 6.4. These measurements indicate that β-lipovitellin is present in the granules in the associated form, and this is certainly true of α-lipovitellin, which does not dissociate except at higher pH (7).

Typical patterns used for measuring sedimentation coefficients and protein concentrations in both solvents are shown in Fig. 2. These include isolated

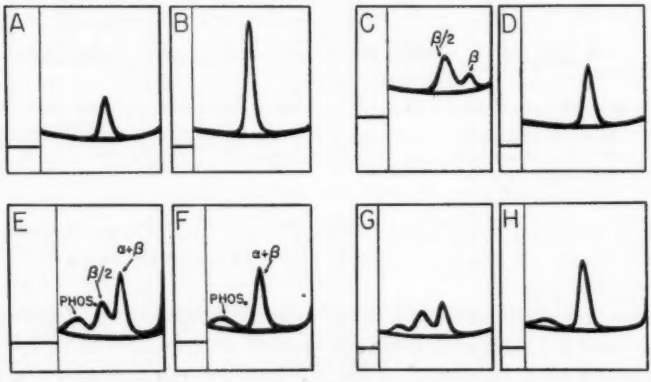

FIG. 2. Ultracentrifuge patterns, after about 60 minutes, of granule proteins at 20° C in solutions of different concentrations. α-Lipovitellin at pH 9.0 (A) and in *M* NaCl (B). β-Lipovitellin at pH 9.0 (C) and in *M* NaCl (D). Synthetic mixture of phosvitin and α- and β-lipovitellin at pH 9.0 (E) and in *M* NaCl (F). Granules with LDF removed, at pH 9.0 (G) and in *M* NaCl (H). Sedimentation from left to right.

α-lipovitellin (A and B), β-lipovitellin (C and D), their mixture with phosvitin in approximately the proportions estimated in the granules (E and F), and the granule proteins from which the LDF had been removed to prevent interference (G and H). Clearly, the β-lipovitellin isolated from the granules dissociates in the same manner as that isolated previously from whole yolk solutions (7), and the patterns obtained from the synthetic mixture are similar to those of the granule proteins.

Sedimentation coefficients at zero concentration are summarized in Table III together with previous results. Little significance can be attached to the differences in the sedimentation rates of the two lipovitellins or between those reported in the two solvents. The present results show that solutions of the granules contain α- and β-lipovitellin and phosvitin. They also suggest that 11 S and 7 S are the most probable values for the associated lipovitellins and for the subunit, respectively. Phosvitin appears to have a higher sedimentation rate in mixtures. This could indicate interaction with lipovitellin, although measurements on the small phosvitin boundaries were difficult.

TABLE III

Sedimentation coefficients ($s^0_{20,w}$ in Svedbergs)

	α-Lipovitellin		β-Lipovitellin			Phosvitin	
	pH 9.0	*M* NaCl	pH 9.0 assoc.	pH 9.0 dissoc.	*M* NaCl	pH 9.0	*M* NaCl
Isolated proteins							
Present work	10.6	10.9	11.0	6.9	10.9	3.0	2.9
Bernardi and Cook (7)	12.0	10.9	11.9*	7.4*	10.4		
Moolenaar (17)	10.8*		10.8*	7.0*		2.7	
Joubert and Cook (16, 22)	11.1†	10.9†				3.3	3.1
Mixed proteins							
As in granules‡	10.6 ($\alpha+\beta$)	10.2 ($\alpha+\beta$)		6.9 ($\beta/2$)		3.6 (phos.)	3.3 (phos.)
Prepared from isolated proteins‡	11.0 ($\alpha+\beta$)	10.9 ($\alpha+\beta$)		6.9 ($\beta/2$)		3.0 (phos.)	3.7 (phos.)

*Values for a mixture of α- and β-lipovitellins.
†Values for a mixture probably rich in α-lipovitellin.
‡Symbols in parentheses identify peaks in Fig. 1 and Fig. 2.

A partial specific volume of 0.545 ml/g was used in calculating the sedimentation coefficients of phosvitin in Table III (16). A sample of β-lipovitellin was found to have a partial specific volume of 0.788 ml/g and this was used for the lipovitellin calculations. The slightly lower value (0.777 ml/g) found by Bernardi and Cook (7) is consistent with the lower lipid and possibly higher phosvitin concentration in their preparation.

Composition of Yolk Granules

The LDF was estimated directly by preparing a solution of the granules of known concentration in 1 *M* sodium acetate or sodium chloride, usually 30 ml of a ca. 2.5% solution. This was then centrifuged at 0° C for 16 hours at 30,000 r.p.m. and the floating layer cut off with the tube slicer and dialyzed against water before the solid was dried to constant weight. Quantitatively the LDF was the most variable constituent in the granules. An average value of 12% is reported in summarizing the composition of the granules (Table V) but its amount in different preparations varied from 10 to more than 20%.

The other granule proteins were estimated from area measurements on ultracentrifuge patterns after the LDF had been removed (see Figs. 2G and 2H). For this reason, and because of the variability in the amount of LDF, their relative amounts have usually been expressed in Tables IV and V as percentages of the total sedimenting proteins.

TABLE IV

Amounts of sedimenting components in yolk granules from different eggs

Yolk	Solvent	Phosvitin (%)	Lipovitellin			Recovery
			Total (%)	α- (%)	β- (%)	Phosvitin + lipovitellin (%)
A	pH 9.0 buffer	18.6	79.8	27	52	98.4
	1 *M* NaCl	19.7	79.4			99.1
B	pH 9.0 buffer	14.3	85.6	26	59	99.9
	1 *M* NaCl	15.7	80.0			95.7
C	pH 9.0 buffer	14.9	85.3	36	50	100.2
	1 *M* NaCl	17.4	82.7			99.9
Average	(1 *M* NaCl)	17		29	52	

The proportions of α- and β-lipovitellin were calculated from the patterns at pH 9.0 on the assumption that the slower β-lipovitellin peak (β/2) represented 74% of the total β-lipovitellin in the dissociated form and the other peak (α + β) the rest of the β-lipovitellin and all the α-lipovitellin. Measurements on isolated β-lipovitellin showed that fresh solutions at pH 9.0 at 20° C were 74% dissociated and that within the range of concentrations used (up to 2.5%) the relative areas of the peaks were constant (19). The degree of dissociation was, however, found to be sensitive to temperature, age of solution, and other factors, some of which could be standardized, but the possibility that it differed from 74% in solutions of the granules introduces an uncertainty into this estimation.

TABLE V

Summary of proportions of proteins in granules

Source of data	Amount of sedimenting proteins		
	Phosvitin (%)	α-Lipovitellin (%)	β-Lipovitellin (%)
Ultracentrifuge from:			
Table IV	17	29	52
Pooled samples	19	31	50
Recovery from columns (18% phosvitin assumed)	—	31	51
Average proportions of:			
Sedimenting granule proteins	18	30	51
Total granule proteins*	16	26	45
Yolk solids†	3.7	6.0	10.3

*LDF = 12% of granules.
†Granules = 23% of yolk solids.

Measurements of synthetic mixtures of known composition showed that the concentration of lipovitellin and phosvitin could be estimated with about 5% error. Values for the proportions of α- and β-lipovitellin were more variable and the error was sometimes 10%.

A few measurements were made on granules from individual eggs and these are given in Table IV. A higher proportion of phosvitin is indicated by measurements in 1 *M* sodium chloride than by those in pH 9.0 buffer, possibly because of the presence of small amounts of the postulated phosvitin complex at pH 9.0. The percentage of phosvitin, however, varied between different eggs, even when allowance is made for incomplete recovery in the second sample.

The average composition of the yolk granules indicated by different methods, most of which are based on pooled yolks from many eggs, is given in Table V. In addition to the estimates from ultracentrifugal measurements, the ratio of α-:β-lipovitellin could be estimated from recoveries from hydroxyapatite columns. The limitations of this method are that the recoveries were never quantitative and were apt to be variable, particularly for α-lipovitellin. Average recoveries were about 65% for α-lipovitellin and 85% for β-lipovitellin. The estimates indicate that phosvitin is 18% of the sedimenting protein, with the remainder made up of α- and β-lipovitellin in the approximate ratio α-:β-: : 1:1.8 ± 0.2.

Discussion

The above results, although incomplete and often approximate, provide the most complete analysis of the yolk granules from hen's egg at present available. As isolated, they contain about 50% water and, on a dry basis, they represent 23% of the yolk solids. Further analysis shows that they are 30% lipid, 70% protein, and contain 0.29% calcium, 0.007% iron, and 2.0% phosphorus. Comparison with analyses of whole yolk shows that the granules contain nearly 70% of the calcium, more than 90% of the protein phosphorus, and 95% of the iron in yolk. Separation of their macromolecular constituents shows that 70%

of the solid is lipovitellin with β-lipovitellin predominating, the rest being phosvitin (16%) and low-density lipoprotein (12%). The amount of the low-density lipoprotein is variable and, although it is a likely contaminant, all attempts at removing it by washing were unsuccessful. It must therefore be concluded, in agreement with others (2), that one or more low-density lipoproteins form an integral part of the granules.

More detailed knowledge of the yolk granules would require additional information on their homogeneity and structure. It is not known whether they are homogeneous in composition or whether, for example, α- and β-lipovitellin are in different sorts of granule. Nothing is known about their structure: they may be merely amorphous agglomerates that are insoluble in yolk because lipovitellin, their major constituent, is insoluble at the yolk pH and ionic strength, and moreover, has the ability to co-precipitate phosvitin (6, 20).

Since the granules dissolve in salt solutions or at mildly alkaline pH, the constituents are evidently held together by ionic or secondary forces. The possibility of covalent bonds' being severed by enzyme action seems remote, since experiments made at 4° C in about 90 minutes gave results similar to those at room temperatures. These experiments also suggest that phosvitin exists in the granules as a complex, which does not, however, bear much relation to the complex assumed by Schjeide and Urist (3, 21) to be the natural form of phosvitin. The presence of both α- and β-lipovitellin in about the usual proportions in the low-temperature experiments suggests that neither is an artifact produced from the other during isolation.

Estimates of the proportions of phosvitin and lipovitellin in terms of the whole egg yolk solids and the relative amounts of α- and β-lipovitellin differ somewhat from those reported earlier from electrophoretic analyses (6, 7). The proportions reported here appear to be more reliable, since inadequate resolution of the electrophoretic components introduced uncertainties. The present study indicates that phosvitin is 3.7% and lipovitellin 16.3% of the yolk solids.

Clearly, the properties of phosvitin and the lipovitellins isolated from the granules are the same as those isolated from solutions of whole egg yolk. New procedures developed for the purification of the lipovitellins reduced their protein phosphorus to 0.50% for α-vitellin and 0.27% for β-vitellin. The higher phosphorus of α-vitellin has therefore been confirmed. The reversible dissociation of β-lipovitellin has also been confirmed. As indicated by Moolenaar (17) this behavior strongly suggests that lipovitellin has an ordered structure more analogous to a globular protein than to a protein–lipid micelle, although a micellar structure is not precluded.

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ELIMINATION OF ASHING IN THE DETERMINATION OF STRONTIUM AND CALCIUM IN LIQUID MILK[1]

JOHN R. BROWN, ANITA A. JARVIS, AND BELLA TIEFENBACH

Abstract

A "non-ashing" analytical procedure for the determination of calcium and strontium in liquid milk is described. Milk was mixed in bulk with Dowex resin, stirred at 78° C, and calcium and strontium eluted from the resin using 6 *N* HCl. The calcium was determined using a buffered oxalate precipitation procedure, followed by the modified Kramer–Tisdall method. Strontium was analyzed using flame spectrophotometry. An average of 79.4% of added strontium was recovered using the "non-ashing" technique. The method has not been tried on the determination of other radionuclides at the present time.

Introduction

Most methods of radionuclide analysis in milk and in other biological products use the technique of ashing for the elimination of organic constituents (4, 5). Such ashing procedures require special equipment, including large muffle furnaces. The "Health and Safety Laboratory Manual of Standard Procedures" (4) recommends, for dry ashing, the evaporation of milk followed by heating of the residue in an electric muffle furnace, at 500° C for 30–35 hours. When wet ashing procedures are used, the sample is treated with hot nitric acid. Both methods are laborious and time-consuming. A rapid ashing procedure has been reported by Grummit and Milton (6); this procedure requires the liquid milk to drip onto the bottom of a silica beaker maintained at a high temperature by a blast burner, while a second flame ignites the gases evolved from the mouth of the beaker. Considerable attention has been focused on finding a method which would eliminate the necessity of ashing samples, and procedures have been published which require no ashing (7, 8). Murty *et al.* have used trichloroacetic acid precipitations to remove the proteins from the milk (8). Our yield determinations, using this method, were not entirely satisfactory. This may be due to the adsorption of some calcium and strontium on the protein precipitate. In the method described below, Dowex resin was used for the elimination of proteins from liquid milk.

Method

Ion-Exchange Column

The ion-exchange column consisted of a glass tube 25 cm long and of 2.5 cm internal diameter. A sintered glass filter of medium porosity was fused to the bottom of the tube, and to the top an inverted 250-ml Erlenmeyer flask was sealed to act as a liquid reservoir. The column and the reservoir were placed

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Contribution from the Department of Physiological Hygiene, School of Hygiene, University of Toronto, Toronto, Ontario.

in a "column-housing" which was a wooden box lined with aluminum. Between the box and its lining, a 500-watt electric heater was placed, the temperature of the interior of the box being maintained at 55° C or 78° C by means of two thermostats, as required.

Milk Preparation

Commercial milk powder from a large bulk source was reconstituted by adding 500 ml of distilled water to 70 g milk powder. The mixture, to which was added 30 mg strontium (as $SrCl_2$), was stirred with a mechanical stirrer.

Resin Preparation

The appropriate amount (1 or 2 g/g milk powder) of Dowex 50W-X12, 200–400 mesh resin (dry weight), was washed in a separate container with 4 *N* ammonium hydroxide until it became basic, then with distilled water until the washing water became neutral. The same resin has been reused for 15 determinations without affecting the yield of calcium and strontium.

The Determination of Strontium and Calcium

The washed resin was mixed with the reconstituted milk, heated to 78° C, and maintained at this temperature for 30 minutes while being agitated with a mechanical stirrer. The mixture was then set aside until the resin settled, when the supernatant fluid was poured off and the remaining resin was washed four times with 1 liter of distilled water at 78° C; each time the supernatant was discarded. The resin was then transferred to the glass ion-exchange column, which was maintained at a temperature of 78° C while the resin wash water drained. Calcium and strontium were eluted at 55° C from the column using 500 ml 6 *N* HCl previously heated to 55° C, the rate of elution being 5 to 6 ml per minute. Instead of an ion-exchange column, a batch-stripping technique can also be employed for this procedure. The washed resin would then be left in the beaker and washed with 500 ml 6 *N* HCl at 55° C.

Qualitative tests were done on the various fractions to determine the presence or absence of proteins. The supernatant fluid poured off from the resin was strongly positive with the biuret reagent. The last two wash waters as well as the 6 *N* HCl eluate were all negative.

Calcium determinations were made using 2.0 ml of the eluate. This was neutralized with 3 ml of 4 *N* ammonium hydroxide and 1.0 ml of Sulkowitch reagent was used to precipitate the calcium (1). It is important that a pH of 4.2 be obtained, for this is optimum for the precipitation of calcium as calcium oxalate. After 30 minutes the precipitate was centrifuged until it was firmly packed in the tube. The supernatant was decanted by inverting the tube quickly and placing it on a filter paper to dry. The precipitate was then mixed thoroughly with 3 ml of 2% ammonium hydroxide, centrifuged, and the supernatant discarded as before. Following this, 2.0 ml of 1 *N* sulphuric acid was added to the precipitate and titration was carried out with 0.1 *N* potassium permanganate at a temperature of 70° C.

Spectrophotometric determinations of strontium were made on the eluate,

which contained 300 μg strontium in a 5-ml aliquot. An internal standard was made containing 3000 mg per liter of strontium. A series of dilutions were then prepared containing 0.2 ml, 0.4 ml, 0.6 ml internal standard, in a 10-ml volumetric flask, which was then filled to the mark with the sample eluate. The emission was observed at 460.7 mμ on the Beckman DU flame photometer using the wavelength of 466 mμ as the "blank" setting.

Results and Discussion

The results discussed in Tables I and II have been obtained with reconstituted skim milk powder. Determinations on liquid whole milk and human milk gave comparable results.

Aliquots of commercial milk powder were reconstituted with distilled water, and determinations of calcium were made, using the "non-ashing" technique described above, and these were compared with similar determinations made using the ashing method described by Mar (6). According to this procedure the milk powder was initially ashed in an 800-ml Vicor (Silica) beaker over a blast burner until gas evolution ceased; a second burner was used over the mouth of the beaker to burn off the gas evolved. The ashing was completed in an electrical muffle furnace at 500–600° C. It was found that when resin and milk powder were used in equal proportions, the ashing technique yielded a better recovery. Doubling the resin quantity in the "non-ashed" samples, however, resulted in the same range of calcium yield as in the ashed samples. The statistical difference between them was not significant. The results are summarized in Table I. When 0.5 g resin was used per gram of milk powder only 8.6 g calcium was found.

TABLE I

Calcium determination in ashed and "non-ashed" milk samples (mg Ca/g milk powder)

Ashed samples	"Non-ashed" samples	
$\frac{\text{Resin}}{\text{Milk powder}} = 1{:}1$	$\frac{\text{Resin}}{\text{Milk powder}} = 1{:}1$	$\frac{\text{Resin}}{\text{Milk powder}} = 2{:}1$
12.1	11.3	12.9
12.7	10.5	13.0
11.7	10.5	12.6
11.7	9.6	12.9
12.5	10.7	12.1
12.4	11.5	13.2
13.1	11.1	
12.9	10.8	
12.1	10.6	
13.1	10.9	
12.7	10.4	
12.5	11.8	
	10.9	
	11.3	
	11.3	
	10.4	
Mean 12.5 ± 0.5*	10.8 ± 0.5*	12.8 ± 0.4*

*Standard deviation.

Determinations of strontium in milk to which known amounts of strontium had been added yielded similar results in both ashed and "non-ashed" samples. Comparable results were obtained when calcium was determined. Strontium-90 analyses were made using ashed and "non-ashed" samples, and a good measure of agreement was found between them. Determinations of natural strontium were not carried out. It was found that the same range of strontium could be obtained in ashed and "non-ashed" samples provided that the resin/milk powder ratio is 2:1 in the "non-ashed" aliquots. The statistical difference between them was not significant (see Table II).

TABLE II

Strontium recovery (%) in ashed and "non-ashed" milk samples

Ashed samples	"Non-ashed" samples	
$\frac{\text{Resin}}{\text{Milk powder}} = 1:1$	$\frac{\text{Resin}}{\text{Milk powder}} = 1:1$	$\frac{\text{Resin}}{\text{Milk powder}} = 2:1$
81.6	68.8	78.0
82.9	74.0	77.9
81.1	66.6	79.9
77.0	66.6	81.8
Mean 80.6 ± 2.5*	69.0 ± 3.5*	79.4 ± 1.8*

*Standard deviation.

Conclusions

Cation-exchange resin is able to remove calcium and strontium from milk without ashing the liquid samples. The procedure outlined is a reliable alternative method to the currently used ashing techniques.

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A VERSATILE HIGH-VOLTAGE PAPER ELECTROPHORESIS APPARATUS AND ITS APPLICATION TO COMPLEX BIOLOGICAL MATERIALS[1]

ARTHUR E. PASIEKA

Abstract

Improvements on a high-voltage paper electrophoresis apparatus are described. The major improvements consist of a simplified cooling system and modification of the cooling plate to accommodate filter paper sheets 24×48 in. in size. Two methods, both employing solid heat exchangers, have been devised for the dissipation of Joule's heat: (*a*) external cooling, employing a Freon 12 circulating coolant and (*b*) no external cooling, but with heat conductance and radiation by means of an aluminum plate and metal rail.

The application of this modified apparatus to the separation of amino acids and peptides from complex biological materials is illustrated.

Introduction

In the past few years, electrophoretic methods have found increasing application in the separation of complex biological materials. This is particularly true of high-voltage techniques, which effect the separation of many low molecular weight compounds within extremely short periods of time (1).

High-voltage paper electrophoresis has been defined by Kickhöfen (2) as that variation employed when gradients exceed 20 volts per cm. The velocity of substance migration in high-voltage separation on filter paper is increased when the voltage is increased but Joule's heat also increases proportionally to the square of the voltage value. Efficient dissipation of this heat is, therefore, essential for experiments carried out with high voltages. A number of methods for removing heat in high-voltage separations are available but at the present time two systems are preferred: those with liquid, and those with solid, heat exchangers (1). In the present study, solid heat exchangers were selected because they permit the use of a wider range of biological separation techniques than do liquid heat exchangers.

The present investigation was undertaken to devise a versatile high-voltage electrophoresis apparatus that would complement paper chromatographic studies on the amino acid metabolism of normal and malignant cells in tissue culture (3–8). This communication reports the development of such an apparatus and illustrates its versatility in the separation of a variety of biological materials.

Materials and Methods

Power Pack and Electrophoretic Units

The power supply used is a commercial product obtained from Radioionics Limited, Montreal, Que. It is operated from a 115-volt a-c. line and has an

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Contribution from the Biochemical Research Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

output of 0 to 12,000 volts d-c. with 0 to 200 milliamperes. The two leads are of high-quality coaxial cable with shielded plugs. Quarter-inch carbon electrodes are used. Buffer vessels constructed from polyethylene or plexiglas were found suitable.

The electrophoretic unit, shown in Fig. 1, consists of an aluminum plate, 1×24×48 in., on a wooden box containing a copper cooling coil resting on fiberglass insulation. The box is 4 inches in internal depth, has a wooden

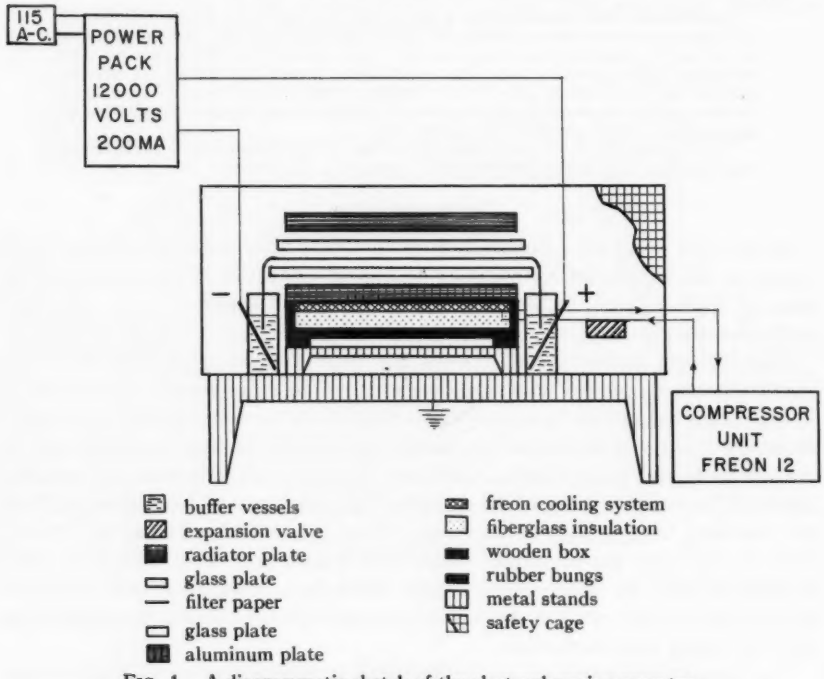


FIG. 1. A diagrammatic sketch of the electrophoresis apparatus.

bottom, and rests on four large rubber bungs, which in turn rest on a 9-in. high metal stand on the bottom of an electrical safety cage. The safety cage is supported on a metal table approximately 2 feet high. The filter paper is placed on the ¼-in. glass plate insulating the aluminum plate. It is covered with a second piece of plate glass or a suitable plastic insulating sheet.

Dissipation of Joule's Heat

(*a*) *By External Cooling*

A compressor and expansion valve utilizing direct expansion of Freon 12 are attached to the apparatus for control of the temperature on the aluminum plate, as shown in Fig. 1. The copper tubes for freon gas lead around the back of the safety cage to two electrically insulated plastic tubes which pass through

the safety cage to the expansion valve and to a series of copper cooling tubes between two copper header pipes. These tubes (A) are shown, in Fig. 2,

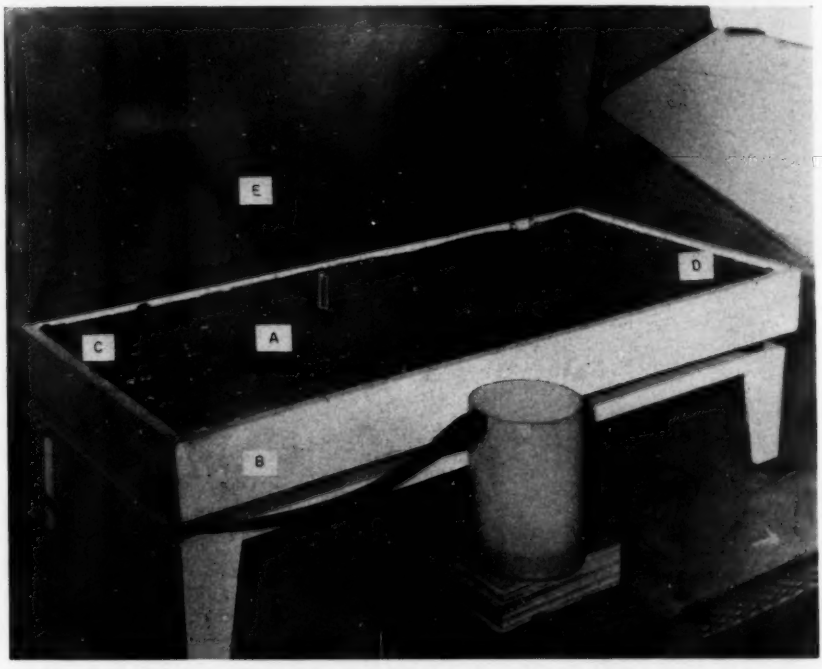


FIG. 2. The Freon 12 cooling system. The two copper tube leads from the compressor enter the fiberglass-insulated wooden box (B) a few inches from the upper right-hand corner as shown. The ¼-in. copper tubes (A) are spaced 1 inch apart over 2-ft width between two copper header pipes (C and D), which are ½ inch at the gas entrance and 1 inch at the return end. The aluminum plate (E) is shown in a raised position as is the safety cage surrounding the whole cooling system.

resting in the wooden box (B) and spaced about an inch apart between the two header pipes (C and D). The aluminum plate (E) then fits snugly on top of these cooling tubes and the wooden box. The cooling is controlled in the same fashion as a domestic refrigerator, and has a range of $-20°$ C to room temperature.

(*b*) *By Conductance and Radiation*

As shown in Fig. 3, a piece of railway steel can be placed on the aluminum plate to substitute for the external cooling system. The rail acts as a radiator of heat and the aluminum plate as a conductor and dissipator. The radiator plate is placed on top of the second piece of glass plate covering the filter paper (Figs. 1 and 3). The rail shown in Fig. 3 is a portion of the standard gauge track used by the Canadian Railways and weighed approximately 29 lb. A

FIG. 3. A 20-in. piece of standard gauge Canadian National Railways steel track used as a radiator plate. The filter paper is moistened with buffer and electrically insulated by two pieces of plate glass sheets. Heavy cotton wicks make contact with moistened paper and hang into the plexiglas electrolyte vessels.

rail approximately 20 inches in length was found satisfactory for use with filter paper strips 24 inches in length and up to 12 inches in width. Copper or brass slabs $\frac{3}{4}$ to 1 inch thick and 20 inches long were also found to be suitable for such papers.

Electrolytes

The volatile electrolyte systems employed at various pH values were mixtures of glacial acetic acid, pyridine, and water similar to those described in the review by Michl (1).

Filter Papers

The filter papers used were Whatman No. 3 for strip separations and Whatman 3MM for two-dimensional separations.

Electrolyte Content of Filter Paper

In order to moisten the filter paper strips with electrolyte in a reproducible manner, a hand-operated clothes wringer, purchased from Dowswell, Lee and Co., Hamilton, Ontario, was used. Electrolyte-soaked filter paper strips were passed through the clothes wringer, which had been adjusted to various

tensions, and the electrolyte content was then determined by weighing each strip in the moist state and after it had been dried in air. The electrolyte content of each strip was expressed as the ratio of these two weights. Strips with varying moisture content were employed in amino acid separations and the most suitable ratio was found to be approximately 1.5 to 1.0. Where the size of the filter paper exceeded the capacity of the clothes wringer, the spraying technique of Gross (9) was used.

Biological Materials

Medium M150 is a completely synthetic medium for tissue cultures developed by Morgan *et al.* (10). It contains approximately 60 organic components, including 21 amino acids, dissolved in a modified Tyrode's solution. The inorganic salt content of this medium is 10 g per liter.

The amino acids from human serum were obtained by precipitating most of the proteins by addition of 95% ethyl alcohol at room temperature until a 60% alcohol solution was obtained. The precipitated proteins were centrifuged, the sedimented material washed twice with small portions of 60% ethanol, and the combined supernatants were then reduced to a small volume over concentrated H_2SO_4 in a vacuum desiccator. Usually, the amino acids from 4 ml of original serum were concentrated to 0.2 ml. The amino acid extraction procedure is essentially the first step of a method developed by Connell and Watson (11) for the extraction of peptides from bacterial cells. This method was found to extract virtually all the amino acids from serum.

The preparation of the lactalbumin fractions has been described previously (8).

Identification of Amino Acids

A series of specific amino acid reactions, described by Block *et al.* (12), was used to identify the separated amino acids when possible. Those amino acids for which there are no described specific reactions were located by loading the samples to be separated with single amino acids and subsequently separating the mixtures.

Procedure

The electrolyte moistened filter paper is placed on the glass plate resting on the aluminum plate, as shown in Fig. 1. The sample to be separated is applied to the filter paper with a micropipette or streaking pipette. The samples may be applied before, or after, placing the filter paper on the glass plate. The filter paper is then covered with a second piece of glass plate or with a plastic sheet such as Saran Wrap (Dow Chemical Co., Sarnia). Contact of the electrolyte-moistened filter paper with the negative and positive electrolyte vessels is made with two wicks moistened with electrolyte. The wicks were made of heavy cotton strips enclosed in dialyzing membranes. Both the cotton strips and the dialyzing membranes were washed thoroughly and extracted with a series of solvents and deionized water before use. The wicks overlapped the filter paper strips by approximately 2 inches at each pole end.

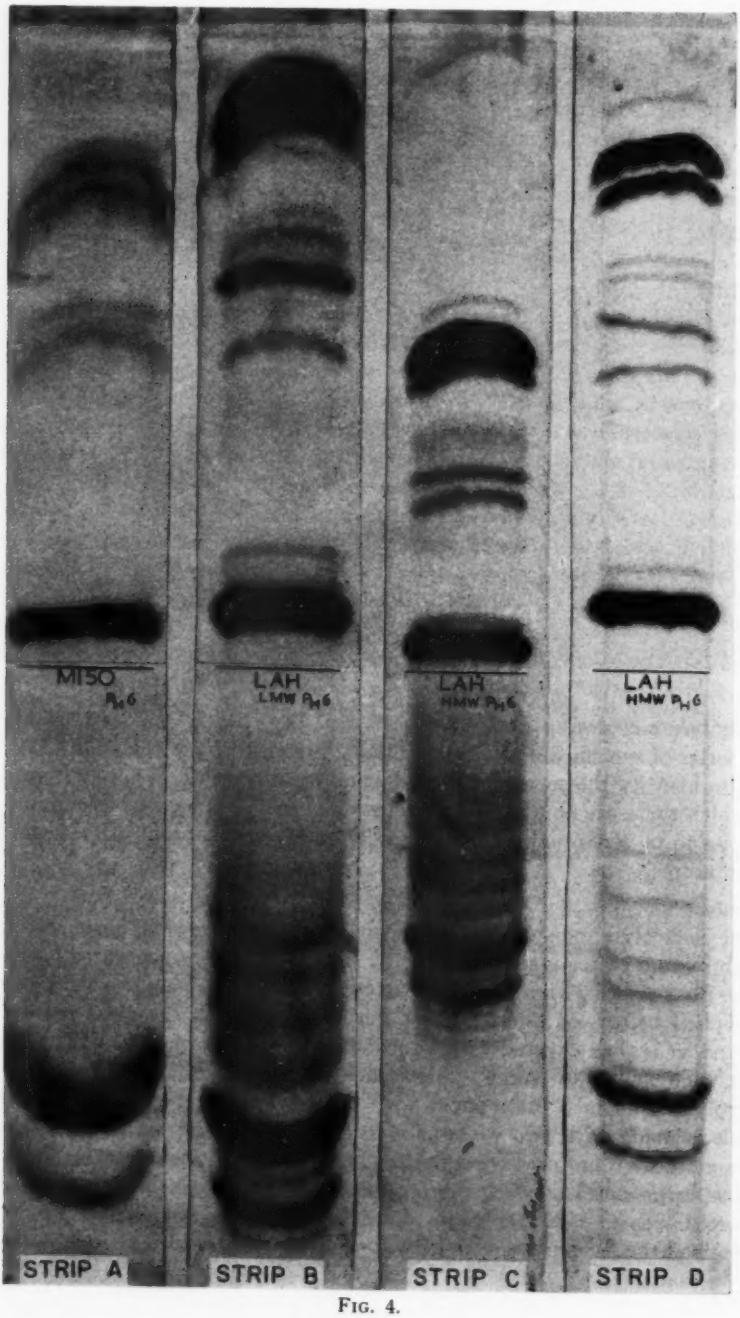


FIG. 4.

Glass rods were then placed on each wick for support (Fig. 3). Separations can then be effected either with or without external cooling of the aluminum plate.

After the electrolyte-moistened filter paper has been placed between the glass plates the safety cage is closed and the main power turned on to the desired voltage. The safety cage is connected through a microswitch to the main power switch so that the high voltage can only be turned on when the cage is in a closed position.

When the run is complete, the filter paper is removed, dried carefully in a chromatography drying oven at 60° to 100° C, and subsequently stained with the appropriate reagent, such as ninhydrin for amino acids. All ninhydrin reactions in this study were carried out by dipping the filter papers in a solution of 0.2% ninhydrin in acetone and subsequently heating these strips 2 to 4 minutes at 100° C in a chromatography drying oven.

Results and Discussion

Electrophoresis with External Cooling

The separation of milligram quantities of amino acids and peptides from typical biological material of high complexity accomplished with external cooling of the aluminum plate is illustrated in Fig. 4. In strip A is shown the separation of amino acids from 2.5 ml of synthetic medium M150. The total dry weight of all components of this sample approximated 35 mg of which the total amino acids make up 2.75 mg and the sodium chloride totals 20 mg. At pH 6.0, the amino acids separated into essentially three main areas: basic amino acids at the top of the filter paper (negative end), neutral amino acids just above the line of application, and acidic amino acids (aspartic and glutamic) at the bottom of the filter paper (positive end).

In strip B is shown the separation of 8 mg dry weight of the low molecular weight (dialyzable) fraction of lactalbumin hydrolyzate. This fraction contains amino acids, amines, and low molecular weight dialyzable peptides and has a very low salt content. Many acidic and basic components are visible and exhibit mobility rates different from those of the 21 amino acids present in M150 (strip A).

Experiments were conducted to determine how the high molecular weight (non-dialyzable) peptide fraction of lactalbumin hydrolyzate behaved under the same conditions of separation and low salt content. This is illustrated on strip C. The speed of migration of this fraction (5 mg dry weight) was found to differ considerably from that of the samples shown in strips A and B. When the non-dialyzable fraction was decreased in concentration to 3 mg (strip D), the migration rate was found to be essentially the same as that of the components of M150 (strip A). It was noted that under comparable conditions, the fastest migration rate of the sample tested was obtained with the low

FIG. 4. The separation of milligram quantities of the high salt synthetic tissue culture medium M150 (strip A) and two fractions of lactalbumin hydrolyzate (strips B, C, and D) on 5×48 in. Whatman No. 3 filter paper.

Conditions: pH 6.0 in the electrolyte, 10 pyridine, 1 acetic acid, and 89 water; 150 volts/inch, 6 ma/inch of width; running time, 110 minutes for each strip.

molecular weight fraction of lactalbumin hydrolyzate (strip B), which had an extremely low salt content. It appeared, therefore, that the concentration of the ninhydrin positive material in the samples was the major factor influencing the migration rate.

In Fig. 4, strips A and D, the bands of electrophoretically separated materials are crescent-shaped. Recent experiments have shown that straighter bands can be obtained if 3-in. margins are left on both sides of the material applied to the filter paper. The crescent shape of the bands is caused mainly by the action of the clothes wringer, which exerts greater pressure and hence removes more electrolyte from the edges than from the center of the paper strip. In addition, more rapid evaporation of the electrolyte occurs from the edges than from the center of the strip during development. As a consequence of these two effects, the materials being separated tend to migrate more rapidly in the center of the paper strip and crescent bands are formed. The provision of margins at each side of the material being separated tends to counteract the uneven electrolyte concentration and evaporation, and relatively straight development bands can be obtained.

Electrophoresis without External Cooling

The most novel feature of the described apparatus is the dissipation of Joule's heat without external cooling but rather through conduction and radiation at room temperature (Fig. 3). In Fig. 5, application of this system to the separation of amino acids at room temperature is shown. In the chromatogram illustrated, the materials have been separated in the first dimension by high-voltage electrophoresis for 15 minutes over a distance of 24 inches. The second dimension has been separated subsequently by descending paper chromatography in a solvent system of *n*-butanol, acetic acid, and water (3). The use of the high-voltage technique for one dimension in this two-dimensional separation reduced the total time required by approximately one-half.

Other one-dimensional high-voltage separations of amino acids at two different pH values are shown in Fig. 6. In all four chromatograms the radiator technique was employed with no external cooling. The first two strips in this figure show the separation of amino acids from medium M150 in two different time periods (7 minutes for strip 1, and 15 minutes for strip 2). These strips illustrate the effectiveness of the band type of separation. Thus, strip 1 shows sharper separations than were obtained by the spot type illustrated in strip 2. Strip 3 shows that very rapid two-dimensional separations of amino acids can be obtained with this technique since separation of the basic amino acids is effected in a total running time of only 10 minutes. Strip 4 illustrates an effective separation of the acidic and basic amino acids from human serum in a running time of 20 minutes.

From a comparison of the electrophoretic patterns illustrated in Figs. 4, 5, and 6 it is evident that essentially similar degrees of separation of complex biological materials can be obtained whether or not external cooling of the

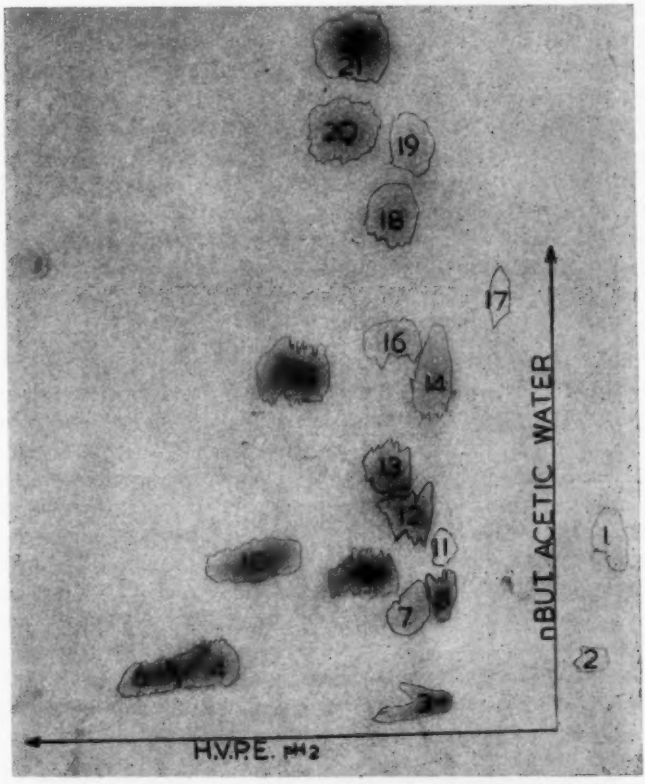


FIG. 5. A two-dimensional separation of amino acids effected by high-voltage electrophoresis in the first dimension and by chromatography in the second dimension, employing completely volatile buffer and solvent systems.

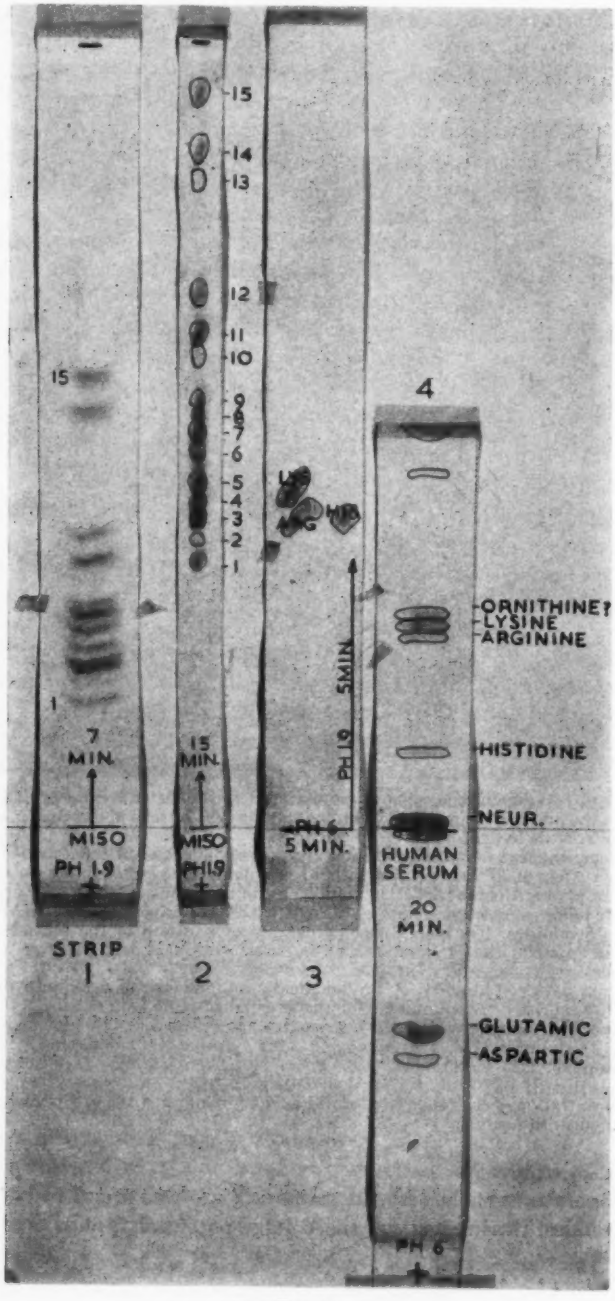
Conditions: the high-voltage separation in the first dimension effected at pH 2.0 in the electrolyte system of 20% acetic acid with no external cooling but with the use of the steel rail radiator plate. A potential of 330 volts/inch and 8 ma/inch of width were applied over a 5-in. wide buffer-moistened section of Whatman 3MM filter paper 24×24 in. Separation time was 15 minutes. The paper chromatographic separation in the second dimension was done in approximately 18 hours with *n*-butanol, acetic acid, and water system according to Pasieka (8).

Separation pattern obtained: (1) cysteic acid? contaminant of cystine in the original mixture, (2) taurine? a contaminant of the original mixture, (3) cystine, (4) histidine, (5) arginine, (6) lysine, (7) glutamine, (8) aspartic acid, (9) serine, (10) glycine, (11) OH-L-proline, (12) glutamic acid, (13) threonine, (14) tyrosine, (15) α-alanine, (16) proline, (17) tryptophan, (18) methionine, (19) valine, (20) phenylalanine, (21) isoleucine, (22) leucine.

The concentration of each separated amino acid approximated 10 μg. Staining agent was 0.2% ninhydrin in acetone.

apparatus is employed.

The temperature reached by the radiator plate was found to be a function of two variables, the intensity of the voltage potential applied, and the dura-


15
14
13
12
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9
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4
3
2
1
15
1
7 MIN.
MISO
PH 1.9
15 MIN.
MISO
PH 1.9
PH 1.9
5 MIN.
PH 6
5 MIN.
4
ORNITHINE?
LYSINE
ARGININE
HISTIDINE
NEUR.
HUMAN SERUM
20 MIN.
GLUTAMIC
ASPARTIC
PH 6
STRIP
1
2
3

FIG. 6.

tion of the electrophoretic run necessary to achieve separations. At potentials (less than 300 volts per inch), the temperature of the plate was not found to increase to any appreciable extent even with runs of longer than 1 hour. When high voltages (greater than 300 volts per inch) were applied, however, measurements indicated that the temperature of the plate rose by 15° C by the end of 1 hour. This increase in temperature did not cause appreciable drying of the filter paper. A period of under 1 hour is normally sufficient to effect the separation of amino acids and peptides from biological materials, but it is recommended that external cooling be applied if runs of longer duration are to be carried out. Subject to this qualification, the simplified system, without external cooling, can be recommended for general use.

Versatility of the Apparatus

For effective one-dimensional separations of amino acids and peptides by high-voltage electrophoresis, Werner and Westphal (13) and Gross (14) have advocated filter paper lengths of 80 cm. In the present study, this length was found to be insufficient to permit the maximum separation of many complex biological materials and, accordingly, efforts were made to increase the capacity of the electrophoretic unit. The apparatus described in this communication has been modified to accommodate filter paper of varying widths and lengths from 24 to 48 inches (60 to 120 cm). This increased size has made possible the electrophoretic separation of a variety of biological materials of high complexity and the use of this technique for preparative separations.

The increased size of filter paper sheets which this apparatus will accommodate makes it convenient to carry out two-dimensional separations directly on the one sheet. Thus, it becomes unnecessary to isolate samples and reapply them to a second sheet for complete two-dimensional development. It is also possible to apply this technique, in conjunction with conventional paper chromatography, as the first or second stage in a two-dimensional separation. A somewhat similar technique has been reported by Katz and coworkers (15), employing a liquid heat exchanger, but this method is limited rather severely by the restrictions of the cooling system. A two-dimensional high-voltage separation of the basic amino acids is illustrated in Fig. 6, strip 3. These

FIG. 6. A series of quick amino acid separations effected with no external cooling, but with the radiator plate, using 24-in. Whatman No. 3 filter paper. All strips were stained with 0.2% ninhydrin in acetone.

Strip 1 shows a 5 μl from 5 ml M150 concentrated to 0.2 ml. The sample was streaked onto the paper and the separation shown effected in 7 minutes at pH 1.9 employing 400 volts/inch and 7 ma/inch in paper width.

Strip 2 shows a 2 μl of 5 ml M150 in 0.2 ml. The sample was applied in a spot and the separation effected in 15 minutes at pH 1.9 employing 400 volts/inch and 15 ma/inch of paper width.

The separation pattern obtained was: (1) trytophan, (2) OH-L-proline, (3) aspartic, tyrosine, (4) phenylalanine, cystine, (5) methionine, proline, glutamic acid, (6) threonine, glutamine, (7) leucine, isoleucine, (8) serine, (9) valine, (10) ?, (11) α-ajanine, (12) glycine, (13) histidine, (14) arginine, (15) lysine.

Strip 3 shows a two-dimensional separation of lysine, arginine, and histidine effected in a total running time of 10 minutes at pH 6.0 in the first dimension and at pH 1.9 in the second dimension employing 300 volts/inch and 15 ma/inch in the first dimension and 500 volts/inch and 15 ma/inch in the second dimension.

Strip 4 shows a 20-minute separation of amino acids from human serum. A 60% alcohol extract from 4 ml serum concentrated to 0.2 ml of which 10 μl volume was applied in a band or streak; 300 volts/inch and 20 ma/inch of paper width.

results were obtained by two separate 5-minute separations, at different pH levels.

The apparatus described in the present communication can also facilitate protein separations on filter paper or on starch gels as described by Block *et al.* (2) and Smithies (6). The separation of proteins on filter paper and on starch gels using this apparatus will be presented in another report.

Acknowledgments

The author is greatly indebted to Dr. J. F. Morgan for his advice and constructive criticism during the preparation of this manuscript. Thanks is also due to the personnel of our shops for assembling various parts of the apparatus. The technical assistance of Mrs. C. MacFarlane for part of this work is acknowledged.

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NOTES

ON THE SYNTHESIS IN VIVO OF LONG-CHAIN UNSATURATED FATTY ACIDS*

D. C. LEEGWATER,† B. M. CRAIG, AND J. F. T. SPENCER

To study the sequence of formation of the unsaturated C_{18} fatty acids, Simmons and Quackenbush (1) fed sucrose-C^{14} to soybean cuttings and determined the rate of increase in specific activity of the fatty acids of the developing seeds. A comparable experiment was carried out by Crombie and Ballance (2) with the fungus *Trichoderma viride*. Both groups of workers observed that oleic acid was quite readily synthesized from the radioactive substrate whereas linoleic acid and linolenic acid became labelled at a much slower rate. They tentatively concluded that oleic acid is the first unsaturated C_{18} fatty acid formed and that the others are probably synthesized subsequently via some dehydrogenation process.

The biosynthesis of lipids in vivo is being studied in this laboratory with the mushroom *Tricholoma nudum* as a test organism (to be published). We wish here to report the results of an experiment similar in design to the ones carried out by the above-mentioned workers. Some of the data obtained indicate, however, that in studies of this type the assumption that the results can be interpreted in terms of the rate and sequence of fatty acid synthesis is open to criticism.

Experimental

Five cultures of *T. nudum* were grown in a basal medium to which were added 2 g ammonium tartrate and 60 g glucose monohydrate per liter (3). After 3 days, the mycelium of each flask was collected by filtration and washed once with distilled water. One culture was dried in a vacuum oven at 55° C, and the others were resuspended in 500-ml Erlenmeyer flasks containing 200 ml of basal medium. To the latter cultures was added uniformly labelled glucose-C^{14} (5.6 μc per flask, specific activity 20 μc/mg). After ½ hour the mycelium of one flask was collected and dried; to each of the remaining flasks was added 4 g inactive glucose in 10 ml water. Further batches of mycelium were harvested and dried at regular intervals. The flasks were continuously shaken during the experiment.

The lipids produced were separated into two groups by extracting dried, ground, mycelium successively with Skelly "F"‡ and methanol.

Analysis of Skelly "F" extracts from inactive material revealed that almost 90% of the lipids of this fraction are triglycerides; small amounts of ergosterol

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†National Research Council of Canada Postdoctorate Fellow, 1959–.
‡Petroleum ether, b.p. 35–58° C (Skelly Oil Company, Kansas City, U.S.A.).

esters, free fatty acids, ergosterol, and some as yet unidentified non-saponifiable compounds are also present. Over 95% of the methanol lipids are phospholipids; phosphatidyl choline (about 60%) and phosphatidyl ethanolamine (about 25%) are the major components; phosphatidyl serine and at least two as yet unidentified phospholipids are minor constituents.

The Skelly "F" and methanol fractions were treated with methanolic HCl. To determine the specific activities, the methyl esters of the fatty acids thus obtained were separated by gas–liquid phase chromatography and counted in an ionization chamber after wet combustion. The results are summarized in Table I (Skelly "F" fraction) and Table II (methanol fraction).

TABLE I

Composition and specific activities in Skelly "F" fraction

Harvest time (hours)	Dry weight mycelium (mg)	Amount of oil (mg)	Spec. act. in mμc/mg methyl ester				Total activity (mμc)
			Palmitic	Stearic	Oleic	Linoleic	
0	1286	257	—	—	—	—	—
½	1287	265	0.25	0.81	0.54	0.16	92
2	1400	274	1.03	1.60	0.98	0.31	220
4	1441	277	1.30	2.05	1.18	0.94	318
24	1860	564	1.19	1.56	1.18	1.35	696
			Composition in wt. % methyl ester*				
			25	8.4	32	30	

*Average values; the composition was almost constant during the experiment. Small amounts of myristic, palmitoleic, and linolenic acids were also present.

TABLE II

Composition and specific activities in methanol fraction

Harvest time (hours)	Total amount of methyl esters (mg)	Spec. act. in mμc/mg methyl ester				Total activity (mμc)
		Palmitic	Stearic	Oleic	Linoleic	
0	33	—	—	—	—	—
½	42	2.24	2.13	3.63	2.43	105
2	42	5.05	4.54	4.34	3.75	166
4	39	2.03	1.53	1.13	3.08	96
24	34	0.37	0.10	0.67	1.20	31
		Composition in wt. % methyl ester*				
		18	3.7	15	59	

*Average values; the composition was almost constant during the experiment. Small amounts of myristic, palmitoleic, and linolenic acids were also present.

Discussion

The data show striking differences in the specific activities of the various fatty acids, both within and between the two fractions. Especially remarkable is that in the beginning of the experiment the labelled fatty acids were incorporated relatively much more rapidly into the methanol lipids than into the Skelly "F"' lipids. By contrast, Crombie and Ballance reported that in their study "analysis of the free fatty acids, phosphatide fatty acids and glyceride

fatty acids showed the same distribution of radioactivity". It is not quite clear, however, whether they were referring to both distribution between and distribution within lipid classes. (Simmons and Quackenbush extracted their material with hexane and most probably studied lipids comparable with our Skelly "F" lipids.)

The main feature of the present investigation is the finding that, in the early stages of the experiment, the rate of incorporation of newly formed, radioactive linoleic acid into the methanol lipids was of the same order as that of oleic acid, whereas, in the Skelly "F" lipids, linoleic acid became labelled much more slowly than oleic acid. This finding is of interest in connection with the problem of the biosynthesis of long-chain unsaturated fatty acids and indicates that the rate of increase in specific activity of a fatty acid in a specified lipid is not necessarily a direct reflection of the actual rate of synthesis of that fatty acid. Although the data for both the Skelly "F" and the methanol fraction are not inconsistent with the hypothesis that there is a precursor–product relation between oleic and linoleic acid, it seems quite possible that the relatively slow incorporation of radioactivity into the linoleic acid of triglycerides, observed in the present study and in the studies previously mentioned, was not the result of a direct conversion of oleic into linoleic acid. In consequence, as long as it has not been established that the unsaturated fatty acids are indeed biosynthetically closely interrelated, it is doubtful whether time – specific activity data of studies of the present type can be considered to represent true precursor–product relations.

It is now being investigated whether a more detailed analysis of the fate of the radioactive substrate (distribution over both lipid and non-lipid fractions) will give a better clue to an understanding of the pathway(s) involved in the synthesis in vivo of linoleic acid and what the significance of the high incorporation of radioactivity into the methanol lipids in the early stages of the experiments might be.

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NOTES TO CONTRIBUTORS

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